

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

VOSSIUS & PARTNER
P.O. Box 86 07 67
D-81634 München
ALLEMAGNE

| | |
|--|---|
| Date of mailing (day/month/year) 20 August 1996 (20.08.96) | IMPORTANT NOTIFICATION |
| Applicant's or agent's file reference K 432 PCT | |
| International application No. PCT/EP95/00859 | International filing date (day/month/year) 07 March 1995 (07.03.95) |

| | | |
|---|-----------------------------|---------------------------|
| 1. The following indications appeared on record concerning: <input type="checkbox"/> the applicant <input type="checkbox"/> the inventor <input checked="" type="checkbox"/> the agent <input type="checkbox"/> the common representative | | |
| Name and Address | State of Nationality | State of Residence |
| | Telephone No. | |
| | Facsimile No. | |
| | Teleprinter No. | |
| 2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: <input checked="" type="checkbox"/> the person <input type="checkbox"/> the name <input type="checkbox"/> the address <input type="checkbox"/> the nationality <input type="checkbox"/> the residence | | |
| Name and Address VOSSIUS & PARTNER P.O. Box 86 07 67 D-81634 München Germany | State of Nationality | State of Residence |
| | Telephone No. | |
| | Facsimile No. | |
| | Teleprinter No. | |
| 3. Further observations, if necessary: An agent has been appointed. | | |
| 4. A copy of this notification has been sent to: <input checked="" type="checkbox"/> the receiving Office <input type="checkbox"/> the designated Offices concerned <input type="checkbox"/> the International Searching Authority <input checked="" type="checkbox"/> the elected Offices concerned <input type="checkbox"/> the International Preliminary Examining Authority <input type="checkbox"/> other: | | |

| | |
|--|--|
| The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35 | Authorized officer H. Zhou Telephone No.: (41-22) 730.91.11 |
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PATENT COOPERATION TREATY

PCT

REC'D 30 MAY 1996

WIPO PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

| | | |
|--|--|---|
| Applicant's or agent's file reference K 432 PCT | FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) | |
| International application No. PCT/EP 95/ 00859 | International filing date (day/month/year) 07/03/1995 | Priority date (day/month/year) 09/03/1994 |
| International Patent Classification (IPC) or national classification and IPC C12N15/50 | | |
| Applicant HOECHST SCHERING AGREVO GmbH et al. | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 5 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consists of a total of 9 sheets.

3. This report contains indications and corresponding pages relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

| | |
|--|---|
| Date of submission of the demand 14/09/1995 | Date of completion of this report 28 MAY 1996 |
| Name and mailing address of the IPEA  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx: 523656 epmu d Fax: (+49-89) 2399-4465 | Authorized officer A. Ury  Telephone No. 844 |



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.

PCT/EP95/00859

I. Basis of the report

1. This report has been drawn up on the basis of (Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

☐ the international application as originally filed.

☒ the description, pages 1-67 _____, as originally filed,
pages _____, filed with the demand,
pages _____, filed with the letter of _____,
pages _____, filed with the letter of _____.

☒ the claims, Nos. _____, as originally filed,
Nos. _____, as amended under Article 19,
Nos. _____, filed with the demand,
Nos. 1-59 _____, filed with the letter of 03.05.96,
Nos. _____, filed with the letter of _____.

☒ the drawings, sheets/fig 1/8-8/8 _____, as originally filed,
sheets/fig _____, filed with the demand,
sheets/fig _____, filed with the letter of _____,
sheets/fig _____, filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages _____.
- ☐ the claims, Nos. _____.
- ☐ the drawings, sheets/fig _____.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Intern. application No.

PCT/EP95/00859

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. STATEMENT

| | | |
|-------------------------------|-------------------|-----|
| Novelty (N) | Claims 1-59 _____ | YES |
| | Claims _____ | NO |
| Inventive Step (IS) | Claims 1-59 _____ | YES |
| | Claims _____ | NO |
| Industrial Applicability (IA) | Claims 1-59 _____ | YES |
| | Claims _____ | NO |

2. CITATIONS AND EXPLANATIONS

The following document has been considered for the purposes of this report:

D1: Plant Molecular Biology, 13 (1989) pp.411-418.

I) Inventive step of the present application lies in the finding that a strong inhibition of the citrate synthase activity in cells of potato plants leads to an inhibition of flower formation in these plants and that increasing the citrate synthase activity in said plant cells also leads to a modified flowering behaviour of these plants, in particular to premature flower formation and to an increased number of flowers (pages 2-3 of the description). This is neither disclosed nor suggested by the cited prior art.

II) D1 which is considered to be the closest prior art document amongst those cited in the International Search Report, discloses the isolation of a cDNA encoding mitochondrial citrate synthase from Arabidopsis



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

thaliana. However, D1 does not prompt the skilled person to search in other plants, other DNA sequences coding for citrate synthase proteins. Nor does D1 suggest the DNA sequences as defined in claim 42.

III) Novelty and inventive step (Article 33.2 and 3 PCT) are therefore acknowledged for the subject-matter of the present set of claims.



VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

- 1) The expression:
 - "essentially identical" in claims 46-48 (even in the light of the definition given page 4 of the description) renders the scope of the claim unclear since said "essentially identical amino acid sequence" has no functional limitation; accordingly, the claims do not satisfy the requirement of Article 6 PCT.
- 2) Claims 43-51 cannot be dependent upon claim 1 for obvious reasons (Article 6 PCT)



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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

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|--|---|---|
| Applicant's or agent's file reference 51103AWOM1XX | FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below. | |
| International application No. PCT/EP 95/ 00859 | International filing date (<i>day, month, year</i>) 07/03/95 | (Earliest) Priority Date (<i>day, month, year</i>) 09/03/94 |
| Applicant HOECHST SCHERING AGREVO GmbH et al. | | |

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. ☐ Certain claims were found unsearchable (see Box I).

2. ☐ Unity of invention is lacking (see Box II).

3. ☒ The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

☒ filed with the international application.

☐ furnished by the applicant separately from the international application,

☐ but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.

☐ Transcribed by this Authority

4. With regard to the **title**, ☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

Figure No. --- ☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/00859

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/50 C12N15/82 C12N9/88 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|--|
| P,X | EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL 14 (4). 1995. 660-666., LANDSCHUETZE V., ET AL. 'Inhibition of flower formation by antisense repression of mitochondrial citrate synthase in transgenic potato plants leads to a specific disintegration of the ovary tissues' see the whole document --- | 1,2,5,8, 11, 14-18, 28,35, 39,40, 42-44, 46,47 |
| P,X | EMBL SEQUENCE DATABASE ACC. NO.X75082 RELEASE 39, 02-05-1994, LANDSCHUETZE V. ET AL., S.TUBEROSUM MRNA FOR MITOCHONDRIAL CITRATE-SYNTHASE see sequence --- -/-- | 1,2,5,8 |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

26 June 1995

Date of mailing of the international search report

Name and mailing address of the ISA

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Authorized officer

Maddox, A



INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 95/00859

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| A | WO-A-93 18154 (AGRONOMIQUE INST NAT RECH) 16 September 1993 see page 12, line 10 - line 24 --- | 19 |
| A | WO-A-93 12239 (ICI PLC) 24 June 1993 see page 9, line 13 - page 11, line 26 --- | 19 |
| A | PLANT MOL BIOL 13 (4). 1989. 411-418. , UNGER E A, ET AL. 'ISOLATION OF A COMPLEMENTARY DNA ENCODING MITOCHONDRIAL CITRATE SYNTHASE FROM ARABIDOPSIS-THALIANA.' see the whole document ----- | 1-10 |



INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 95/00859

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO-A-9318154 | 16-09-93 | FR-A- 2688228 | 10-09-93 |
| | | EP-A- 0629242 | 21-12-94 |
| | | FI-A- 944042 | 12-10-94 |
| | | NO-A- 943266 | 01-11-94 |
| ----- | | | |
| WO-A-9312239 | 24-06-93 | AU-B- 3165793 | 19-07-93 |
| ----- | | | |





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|--|--|-----------|---|
| (51) International Patent Classification ⁶ : C12N 15/50, 15/82, 9/88, 5/10, A01H 5/00 | | A1 | (11) International Publication Number: WO 95/24487 |
| | | | (43) International Publication Date: 14 September 1995 (14.09.95) |
| (21) International Application Number: PCT/EP95/00859 | | | (81) Designated States: AU, CA, HU, JP, KR, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| (22) International Filing Date: 7 March 1995 (07.03.95) | | | |
| (30) Priority Data: P 44 08 629.6 9 March 1994 (09.03.94) DE P 44 35 366.9 22 September 1994 (22.09.94) DE P 44 38 821.7 19 October 1994 (19.10.94) DE | | | |
| (71) Applicant (for all designated States except US): HOECHST SCHERING AGREVO GMBH [DE/DE]; Miraustrasse 54, D-13476 Berlin (DE). | | | |
| (72) Inventors; and (75) Inventors/Applicants (for US only): MÜLLER-RÖBER, Bernd [DE/DE]; Siemensstrasse 71, D-12247 Berlin (DE). LAND-SCHÜTZE, Volker [DE/DE]; Blücherstrasse 55, D-10961 Berlin (DE). LA COGNATA, Ursula [DE/DE]; Wiesbadener Strasse 14, D-12161 Berlin (DE). | | | |
| (74) Common Representative: HOECHST SCHERING AGREVO GMBH; Miraustrasse 54, D-13476 Berlin (DE). | | | |
| (54) Title: PROCESSES FOR INHIBITING AND FOR INDUCING FLOWER FORMATION IN PLANTS | | | |
| (57) Abstract <p>Processes for inhibiting flower formation and processes for inducing flower formation in plants, and processes for improving the storage capability of storage organs of useful plants and processes for reducing the sprouting of tubers in tuberous plants are described. Also described are DNA sequences which modify the activity of the citrate synthase of the plant upon integration into a plant genome, plasmids which contain these DNA sequences and transgenic plants in which modifications in the activity of the citrate synthase are brought about by introducing the DNA sequences. The described DNA sequences are sequences from <i>Solanum tuberosum</i>, <i>Nicotiana tabacum</i> and <i>Beta vulgaris</i> which code for the enzyme citrate synthase. The invention also describes transgenic potato plants in which an inhibition of flower formation, a reduction in the storage losses of the tubers and a reduction in the sprouting of the tubers comes about because of an inhibition of the citrate synthase activity, and transgenic potato plants in which a premature induction of flower production comes about because of the over-expression of a citrate synthase.</p> | | | |

Processes for inhibiting and for inducing flower formation in plants

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The present invention relates to processes for inhibiting flower formation and processes for inducing flower formation in plants, and to processes for improving the storage capability of storage organs of useful plants, and to processes for
10 reducing the sprouting of tubers in tuberous plants. The present invention also relates to DNA sequences which code for plant citrate synthases and to new plasmids containing these DNA sequences, which, upon integration into a plant genome, modify the activity of the citrate synthase in the plant, and
15 to transgenic plants in which modifications in the activity of the citrate synthase are brought about by introducing these DNA sequences.

Because of the continuously increasing demand for food, which
20 results from the constantly growing world population, one of the tasks of biotechnology research is to endeavour to increase the yield of useful plants. One possibility of achieving this consists e.g. of modifying the flowering behaviour of agriculturally useful plants. Increasing the number of flowers
25 is for example desirable with plants whose flowers, fruits or seeds are used agriculturally. Premature flower formation leads to a shortening of the period between sowing and flowering and can thus permit the cultivation of plants in climatic regions with shorter vegetation periods, or the application of two
30 sowings within one vegetation period. Inhibiting flower formation can be advantageous in plants which multiply in predominantly vegetative manner, and can lead to an increased deposition of stored substances in storage organs. One example of such an agriculturally useful plant is the potato.

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Targeted modification of the flowering behaviour in plants has however as yet not been possible since the process of inducing flower formation in plants is not yet very well understood as



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a whole. Various substances such as e.g. carbohydrates, cytokinins, auxin, polyamines and calcium are discussed as inducers of flower formation. Overall, however, the impression is created that flowering induction is a complex process in which several factors interact which have not as yet been unequivocally identified (Bernier et al. (1993) Plant Cell 5:1147-1155).

To date, chemical substances have as a rule been used to modify flowering behaviour. Thus, it is e.g. known that inhibiting flower formation in the case of sugar cane, which leads to a considerable increase in the sugar yield, can be achieved by the exogenous application of different synthetic growth regulators (monuron, diuron, diquat). The use of such synthetic substances is, however, generally associated with a high expenditure and environmental risks which are difficult to assess.

It therefore appears desirable to provide processes which permit a targeted modification of the flowering behaviour, in particular inhibition or induction of the flower formation, in the case of various useful plants, whilst avoiding the use of synthetic substances.

It is therefore the object of the present invention to provide processes which permit plants to be produced whose flowering behaviour is modified, in particular plants which are inhibited in their flower formation, or plants which display premature flower formation and an increased amount of flowers.

The present invention describes genetic engineering processes in which a change occurs in the flowering behaviour of plants because of the modification of the activity of an enzyme which is involved in respiratory processes in the cells.

It was surprisingly found that a strong inhibition of the citrate synthase activity in cells of potato plants leads to a



complete inhibition of flower formation in these plants, and that increasing the citrate synthase activity in cells of transformed potato plants also leads to a modified flowering behaviour of the plants, in particular to premature flower
5 formation and to an increased number of flowers.

To produce plants with a reduced citrate synthase activity, DNA sequences which code for enzymes with the enzymatic activity of a citrate synthase were isolated from different plant species.
10 These are DNA sequences from plants of the *Solanaceae* family, in particular from *Solanum tuberosum* and *Nicotiana tabacum*, and sequences from plants of the *Chenopodiaceae* family, in particular from sugar beet (*Beta vulgaris*).

A subject of the invention are therefore DNA sequences from
15 plants of the *Solanaceae* family, in particular the species *Solanum tuberosum* and *Nicotiana tabacum*, and of the *Chenopodiaceae* family, in particular the species *Beta vulgaris*, which code for enzymes having the enzymatic activity of a citrate synthase, and which, after integration into a plant
20 genome, permit the formation of transcripts by which an endogenous citrate synthase activity can be suppressed, or the formation of transcripts by which citrate synthase activity in the cells can be increased. The invention relates in particular to DNA sequences which code for a protein having one of the
25 amino sequences given in Seq ID No. 1, Seq ID No. 2 or Seq ID No. 3, or for a protein having an essentially identical amino acid sequence, and to DNA sequences which have one of the nucleotide sequences shown in Seq ID No. 1, Seq ID No. 2 or Seq ID No. 3, or an essentially identical nucleotide sequence. The
30 invention also relates to derivatives of the sequences shown in Seq ID Nos. 1-3 which can be derived from these by insertion, deletion, substitution of one or more nucleotides or by recombination, and which code for proteins having the enzymatic activity of citrate synthase.

35 Recombinant DNA molecules, e.g. plasmids, and bacteria containing these DNA sequences or sections or derivatives



thereof are also a subject of the invention.

The term "essentially identical" in relation to DNA and amino acid sequences means that the sequences in question have a high degree of homology and that there is functional and/or structural equivalence between the DNA sequences or amino acid sequences concerned. A high degree of homology is understood to be a sequence identity of at least 40 %, preferably above 60 % and particularly preferably above 80 %. Sequences which are homologous to the sequences according to the invention and differ from the DNA sequence or amino acid sequence according to the invention at one or more positions are as a rule variations or derivatives of this sequence which represent modifications which perform the same function. They can however also be naturally occurring variations, for example sequences from other organisms, or mutations, where these mutations may have been caused naturally or were introduced through targeted mutagenesis. The variations can also be synthetically produced sequences.

The proteins coded by the different variants of the DNA sequence according to the invention have certain common characteristics. These may include e.g. enzyme activity, immunological reactivity, conformation etc., and physical properties such as e.g. the mobility gel electrophoreses, chromatographic behaviour, sedimentation coefficients, solubility, spectroscopic properties, stability etc.

It was found that an inhibition of flower formation occurs in transformed plants when DNA sequences which code for a citrate synthase are introduced into plant cells and expressed in anti-sense orientation, which causes the citrate synthase activity in the cells to be reduced.

Within the scope of the present invention, inhibiting flower formation means that the transformed plants either no longer develop any flowers at all, develop fewer flowers than non-transformed plants or that some flowers do form but they do not



develop into functional flowers. Inhibiting flower formation also means that the plants do indeed develop flowers, but that the latter are sterile and do not lead to the formation of seeds or fruits, or are capable of functioning to only a limited extent and lead to the formation of fewer seeds compared with wild-type plants. In particular, inhibiting flower formation means that male sterile flowers are formed or flowers in which the male reproductive organs form fertile pollen only to a small degree. The term means also that from the plants are formed flowers in which the female reproductive organs are absent, are not functional or are reduced in size compared with wild-type plants.

Inhibiting flower formation also means that transformed plants, if they flower, flower later than non-transformed plants, as a rule several days later, preferably one to several weeks later, in particular 2 to 4 weeks later.

A subject of the invention is therefore the use of DNA sequences which code for a citrate synthase for inhibiting flower formation in plants, and the use of such sequences for the expression of a non-translatable mRNA which prevents the synthesis of endogenous citrate synthases in the cells.

The present invention also relates to a process for inhibiting flower formation in plants, characterized in that the citrate synthase activity in the cells of the plants is reduced, whereby this reduction is achieved preferably by inhibiting the expression of DNA sequences which code for citrate synthases.

Particularly preferred are processes in which flower formation inhibition is achieved by inhibiting the expression of endogenous citrate synthase genes through the use of *anti-sense* RNA.

The present invention relates in particular to processes for inhibiting flower formation in plants, characterized in that



- a) a DNA which is complementary to a citrate synthase gene present in the cell is stably integrated into the genome of a plant cell,
- 5 b) this DNA is expressed constitutively or is inducible due to the combination with suitable elements controlling the transcription,
- 10 c) the expression of endogenous citrate synthase genes is inhibited because of an *anti-sense* effect and
- d) plants are regenerated from the transgenic cells.

The expression of a DNA which is complementary to a citrate synthase gene present in the cell is as a rule achieved by
15 integrating into the genome of the plants a recombinant double-stranded DNA molecule comprising an expression cassette having the following constituents and expressing it:

- 20 A) a promoter functional in plants,
- B) a DNA sequence coding for citrate synthase which is fused to the promoter in *anti-sense* orientation, so that the non-coding strand is transcribed, and if
25 necessary
- C) a signal functional in plants for the transcription termination and polyadenylation of an RNA molecule.

30 Such DNA molecules are also a subject of the invention. The present invention provides such DNA molecules which contain the described expression cassettes in the form of the plasmid pKS-CSa (DSM 8880) which comprises the coding region for citrate synthase from potatoes, and of the plasmid TCSAS (DSM 9359)
35 which comprises the coding region of citrate synthase from tobacco, the composition of which is described in Examples 3



and 8 respectively.

In principle, any promoter active in plants can be used as the promoter. The promoter is to ensure that the chosen gene is
5 expressed in the plant. It is possible to use both those promoters which guarantee a constitutive expression in all tissues of the plant, such as e.g. the 35S promoter of the cauliflower mosaic virus, and those promoters which guarantee expression only in a certain tissue, at a certain time in plant
10 development or at a time determined by external influences. The promoter can be homologous or heterologous in relation to the transformed plant.

The use of tissue-specific promoters represents a preferred subject of the invention.

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The DNA sequence which codes for a protein having the enzymatic activity of a citrate synthase can, in principle, originate from any chosen organism, preferably from plants. The sequence used originates preferably from the plant species which is used
20 for the transformation, or from a closely related plant species.

A preferred embodiment of the process discussed above provides that a DNA sequence which originates from a plant of the Solanaceae family or the Chenopodiaceae family, in particular
25 from *Solanum tuberosum*, *Nicotiana tabacum* or *Beta vulgaris* is used for the DNA sequence which codes for a citrate synthase. Particularly preferred embodiments provide for the use of a DNA sequence which codes for a protein having one of the amino acid sequences given in SeqID No.1, SeqID No.2 or SeqID No.3 or an
30 essentially identical amino acid sequence, in particular a DNA sequence which is identical or essentially identical to one of the DNA sequences given in SeqID No. 1, SeqID No. 2 or SeqID No. 3.

Also, using standard processes and the already known DNA
35 sequences which code for citrate synthases, other DNA sequences can be isolated from any organisms, preferably plants which



code for proteins having the enzymatic activity of a citrate synthase. These sequences can also be used in the processes according to the invention.

- 5 The *anti-sense* orientation of the coding DNA sequence given in B) in relation to the promoter causes a non-translatable mRNA to form in the transformed plant cells which prevents the synthesis of an endogenous citrate synthase.
- 10 Instead of the complete DNA sequences according to the invention given in SeqID No. 1, SeqID No.2 and SeqID No. 3, partial sequences thereof can also be used for the *anti-sense* inhibition. Sequences up to a minimum length of 15 bp can be used. However, an inhibiting effect is not excluded when shorter sequences are used either. Longer sequences between 100
- 15 and 500 base pairs are preferably used, for an efficient *anti-sense* inhibition, sequences having a length above 500 base pairs are used in particular. As a rule, sequences are used which are shorter than 5000 base pairs, preferably sequences which are shorter than 2500 base pairs.
- 20 It is also possible to use DNA sequences which have a high degree of homology to the DNA sequences according to the invention, but which are not completely identical, in the process according to the invention. The minimum homology should be greater than approx. 65 %. The use of sequences having
- 25 homologies between 95 and 100 % is to be preferred.
- DNA sequences can also be used which result from the sequences shown in SeqID No. 1, SeqID No. 2 or SeqID No. 3 by insertion, deletion or substitution without the inhibiting effect of the *anti-sense* sequence thereby being destroyed.
- 30 The DNA fragments used for the construction of *anti-sense* constructs can also be synthetic DNA fragments which were produced using current DNA synthesis techniques.

The plants obtainable from the described process are also a

35 subject of the invention, which are characterized in that they display a reduced citrate synthase activity in the cells as a



result of the expression of an *anti-sense* RNA which is complementary to DNA sequences which code for a protein having the enzymatic activity of a citrate synthase. Such plants are also characterized in that they contain an expression cassette
5 stably integrated into the genome, which comprises the following sequences:

- A) a promoter functional in plants,
- 10 B) a DNA sequence coding for citrate synthase which is fused to the promoter in *anti-sense* orientation, so that the non-coding strand is transcribed, and if necessary
- C) a signal functional in plants for the transcription
15 termination and polyadenylation of an RNA molecule.

The plants are preferably the plants given above.

As is described in the embodiments taking the potato as an
20 example, there occurs in potato plants, because of the reduction in the citrate synthase activity by means of an *anti-sense* effect, an inhibition of flower formation in transformed plants. In particular, transformed potato plants display more or less drastic phenotypes depending on the degree of reduction
25 in the citrate synthase activity. A marked reduction in the citrate synthase leads to the complete inhibition of flower formation. Plants with a less marked inhibition do produce buds but these are not developed to functional flowers. Plants can also be produced which develop flowers, but whose female
30 reproductive organs are not functional.

Similar effects are observed with transgenic tobacco plants which show a reduction in the citrate synthase activity. Flowers are developed here also whose female reproductive organs are greatly reduced in size.

35 The inhibition of flower formation via the reduction in the citrate synthase activity is not however only of interest for



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potatoes or tobacco, but should be of wider significance for plant breeding and agriculture. E.g. the possibility can be cited of achieving a chronologically determined flower induction or inhibition by combining the DNA sequences according to the invention with exogenously regulatable control elements. This can play a role in the prevention of frost damage.

The processes according to the invention can be used both on dicotyledons as well as on monocotyledons. Plants which are of particular interest are useful plants such as types of grain (e.g. rye, wheat, corn, oats, barley, maize, rice etc.), types of fruit (e.g. apricots, peaches, apples, plums etc.), types of vegetable (e.g. tomatoes, broccoli, asparagus etc.), ornamental plants or other economically interesting types of plants (e.g. potatoes, tobacco, rapeseed, soya beans, sunflowers, sugar cane etc.).

The use of the present invention in particular with sugar beet is of particular interest, since here "shooting" can be prevented by inhibiting flower formation. Since shooting is induced by low temperatures, the seeds are planted relatively late (in April/May) in order to prevent shooting. By inhibiting the citrate synthase in sugar cane, a reduction in shooting would be achieved. This permits the sugar beet seeds to be sown earlier which then leads to an increased yield because of the extended vegetation period.

In addition to inhibiting flower formation, in transformed potato plants which display a reduced citrate synthase activity in the cells, a reduced sprouting of the tubers and a reduced respiration in cells of the tubers was observed, compared with non-transformed plants. This leads to lower storage losses and an improved storage capability of the tubers. The process according to the invention is therefore also suitable for producing plants with an improved storage capability of the storage organs, whereby improved storage capability is understood within the context of this invention to mean that



the stored storage organs of transformed plants show smaller losses of fresh and dry weight after a period of storage, compared with those of non-transformed plants. Storage organs are understood to be typical harvestable organs of plants, such as seeds, fruits, tubers and beets.

The process is suitable in particular for producing transgenic potato plants whose tubers have an improved storage capability, smaller storage losses and reduced sprouting of tubers compared with wild-type plants. Reduced sprouting of tubers means that the tubers of transformed plants form sprouts which have a lower fresh and dry weight compared with sprouts of non-transformed plants. The commercial benefits of these effects are obvious.

A subject of the invention are therefore also processes for improving the storage capability of storage organs in plants, characterized in that the citrate synthase activity in the cells of the plants is reduced, this reduction preferably being achieved by inhibiting the expression of DNA sequences which code for citrate synthases.

Particularly preferred are processes in which the citrate synthase activity is reduced by inhibiting the expression of endogenous citrate synthase genes through the use of anti-sense RNA.

The present invention relates in particular to processes for improving the storage capability of storage organs in plants, characterized in that

- a) a DNA which is complementary to a citrate synthase gene present in the cell is stably integrated into the genome of a plant cell,
- b) this DNA is expressed constitutively or inductively by combination with suitable elements controlling the



transcription,

- c) the expression of endogenous citrate synthase genes is inhibited by an *anti-sense* effect and
- d) plants are regenerated from the transgenic cells.

5

Such processes can be used on all types of plants which develop storage organs, preferably on agricultural useful plants and particularly preferably on types of grain (rye, barley, wheat, maize, rice etc.), types of fruit, types of vegetable, on
10 plants which develop tubers such as e.g. potatoes or manioc, and on plants which develop beet as storage organs, in particular sugar beet.

A subject of the invention are also processes for the
15 production of transgenic tuberous plants whose tubers display reduced sprouting, characterized in that the citrate synthase activity in the cells of the plants is reduced, this reduction preferably being achieved by inhibiting the expression of DNA sequences which code for citrate synthases.

20

Particularly preferred are processes in which the reduction in the citrate synthase activity is achieved by inhibiting the expression of endogenous citrate synthase genes through the use of *anti-sense* RNA.

25

The present invention relates in particular to processes for the production of transgenic tuberous plants whose tubers display reduced sprouting, characterized in that

- 30 a) a DNA which is complementary to a citrate synthase gene present in the cell is stably integrated into the genome of a plant cell,
- b) this DNA is expressed constitutively or inductively by combination with suitable elements controlling the
35 transcription,
- c) the expression of endogenous citrate synthase genes is



inhibited because of an *anti-sense* effect and
d) plants are regenerated from the transgenic cells.

Such processes can preferably be used for the production of
5 transgenic potato and manioc plants.

What has already been stated above for the process for
inhibiting flower production also applies to the various
possibilities in the embodiments of the given processes, in
10 particular for the choice and length of the DNA sequence used
which codes for a citrate synthase, and to the choice of
promoter.

As an alternative to reducing the citrate synthase activity in
15 plant cells using an *anti-sense* effect, the reduction can also
be achieved by introducing a DNA sequence which codes for a
ribozyme which specifically cleaves transcripts of endogenous
citrate synthase genes in endonucleolytic manner. Ribozymes are
catalytically active RNA molecules which are able to cleave RNA
20 molecules at specific target sequences. Using genetic
engineering methods it is possible to modify the specificity of
ribozymes. There are different classes of ribozymes. For
practical application with the aim of cleaving the transcript
of a certain gene in targeted manner, representatives of two
25 different groups of ribozymes are preferably used. The first
group comprises ribozymes which are to be assigned to the
Group I-intron-ribozymes. The second group comprises ribozymes
which have as a characteristic structural feature a so-called
"hammerhead" motif. The specific recognition of the target RNA
30 molecule can be modified by changing the sequences which flank
this motif. Via base pairing with sequences in the target
molecule, these sequences determine the site at which the
catalytic reaction and therefore cleavage of the target
molecule takes place. Since the sequence requirements for an
35 efficient cleavage are extremely low, it therefore appears
possible in principle to develop specific ribozymes for



practically any RNA molecule.

Genetically modified plants whose citrate synthase activity is drastically reduced can therefore also be produced by
5 introducing and expressing a recombinant double-stranded DNA molecule in plants which comprises:

- a) a promoter functional in plants
- 10 b) a DNA sequence which codes for a catalytic domain of a ribozyme and which is flanked by DNA sequences which are homologous to sequences of the target molecule, and, if necessary,
- 15 c) a signal, functional in plants, for the transcription termination and polyadenylation of an RNA molecule.

Coming into consideration for the sequence under b) are e.g. the catalytic domain of the satellite DNA of the SCMo virus
20 (Davies et al., 1990, Virology, 177:216-224) or that of the satellite DNA of the TobR virus (Steinecke et al., 1992, EMBO J., 11:1525-1530; Haseloff and Gerlach, 1988, Nature 334:585-591).

The DNA sequences which flank the catalytic domain are formed
25 of DNA sequences which are homologous to the sequences of endogenous citrate synthase genes.

The same as was already stated above for the construction of *anti-sense* structures applies to the sequences given in a) and
c).

30

A further aspect of the present invention consists in the expression of DNA sequences which code for proteins having the enzymatic activity of a citrate synthase in *sense* orientation in plant cells in order to increase the citrate synthase
35 activity. For this, a DNA sequence coding for citrate synthase is fused in *sense* orientation to a promoter, i.e. the 3'-end of



the promoter is linked to the 5'-end of the coding DNA sequence. This leads to the expression of an mRNA coding for citrate synthase and consequently to an increased synthesis of this enzyme.

5

It was now surprisingly found that, as a result of the increase in the citrate synthase activity in cells of transformed plants, a modification of flowering behaviour occurs compared with non-transformed plants. In particular, flower formation is
10 induced. Within the scope of the present invention, the following are understood by this:

a) a premature flower formation (this means in this connection that transformed plants flower earlier compared with non-transformed plants, as a rule a few days earlier, preferably
15 one to several weeks earlier) and/or

b) an enhanced flower formation (this means in this connection that transformed plants produce more flowers, preferably at least 10 % more flowers, compared with non-transformed plants).

20 Such an effect is desirable in a series of cultivated and useful plants such as types of vegetables, e.g. tomatoes, paprika, pumpkin, melons, gherkins, courgettes, rapeseed, types of grain, maize or cotton and in various ornamental plants.

25 A further subject of the present invention is therefore the use of DNA sequences which code for proteins having the enzymatic activity of a citrate synthase, for inducing flower formation in plants, and processes for inducing flower formation in plants, characterized in that the citrate synthase activity in
30 the cells of the plants is increased. The citrate synthase activity is increased preferably by introducing a recombinant DNA molecule into plant cells which comprises the coding region for a citrate synthase and which leads to the expression of a citrate synthase in the transformed cells.

35



Such processes preferably comprise the following steps:

- a) stably integrating a DNA, which is of homologous or heterologous origin and which codes for a protein having citrate synthase activity, into the genome of a plant cell,
- b) expressing this DNA constitutively or inductively by combining with suitable elements controlling the transcription,
- c) thereby increasing the citrate synthase activity in the cells and
- d) regenerating plants from the transgenic cells.

The expression of a DNA which codes for a protein having the enzymatic activity of a citrate synthase is as a rule achieved by integrating a recombinant double-stranded DNA molecule comprising an expression cassette having the following constituents into the genome of the plants and expressing it:

- A) a promoter functional in plants,
- B) a DNA sequence coding for citrate synthase which is fused to the promoter in *sense* orientation, and if necessary
- C) a signal functional in plants for the transcription termination and polyadenylation of an RNA molecule.

Such DNA molecules are also a subject of the invention. The present invention provides those DNA molecules which contain such expression cassettes, in the form of the plasmid pHS-mCS, which comprises the coding region for citrate synthase from *S. cerevisiae*, and of the plasmid pEC-mCS, which comprises the



coding region of citrate synthase from *E. coli*.

The DNA sequences given in point a) of the process, which code for citrate synthase, can be of both homologous or native and
5 heterologous or foreign origin in relation to the host plant to be transformed. They can be of pro- as well as eukaryotic origin. DNA sequences coding for citrate synthase from the following organisms are for example known: *Bacillus subtilis* (U05256 and U05257), *E. coli* (V01501), *R. prowazekii* (M17149),
10 *P. aeruginosa* (M29728), *A. anitratum* (M33037) (see Schendel et al. (1992) Appl. Environ. Microbiol. 58:335-345 and references contained therein), *Haloferax volcanii* (James et al. (1992) Biochem. Soc. Trans. 20:12), *Arabidopsis thaliana* (Z17455) (Unger et al. (1989) Plant Mol. Biol. 13:411-418), *B. coagulans*
15 (M74818), *C. burnetii* (M36338) (Heinzen et al. (1991) Gene 109:63-69), *M. smegmatis* (X60513), *T. acidophilum* (X55282), *T. thermophila* (D90117), pig (M21197) (Bloxham et al. (1981) Proc. Natl. Acad. Sci. 78:5381-5385), *N. crassa* (M84187) (Ferea et al. (1994), Mol. Gen. Genet. 242:105-110) and *S. cerevisiae*
20 (Z11113, Z23259, M14686, M54982, X00782) (Suissa et al. (1984) EMBO J. 3:1773-1781). The numbers in brackets give in each case the accession numbers under which these sequences are accessible in the GenEMBL data bank. The sequences can be isolated from the said organisms by means of current molecular
25 biology techniques or they can be produced synthetically.

A preferred embodiment of the process according to the invention provides for the use of DNA sequences which code for citrate synthases which, compared with citrate synthases
30 normally occurring in plants, are deregulated or unregulated, i.e. are not regulated in their enzymatic activity by regulation mechanisms which influence the activity of the citrate synthase in plant cells. Deregulated means in particular that these enzymes are not inhibited to the same
35 degree by the inhibitors or activated by the activators which normally inhibit or activate plant citrate synthases.



Unregulated citrate synthases are understood within the scope of this invention to be citrate synthases which are not subject to regulation by inhibitors or activators in plant cells.

5 Prokaryotic, in particular bacterial, DNA sequences are preferably used which code for citrate synthases since they have the advantage that the proteins which are coded by these sequences are subject to no regulation or only weak regulation in plant cells. It is thereby possible that an increase in
10 citrate synthase activity occurs through expression of an additional citrate synthase in plant cells.

In a preferred embodiment of the described process, DNA sequences from *E. coli* are used which code for a protein with
15 citrate synthase activity, in particular the gene *glt A* (Sarbjit et al., 1983, Biochemistry 22:5243-5249).

A further preferred embodiment of the process according to the invention provides for the use of DNA sequences from
20 *Saccharomyces cerevisia* which code for citrate synthase, in particular the use of the DNA sequences described by Suissa et al. (1984, EMBO J. 3:1773-1781).

In cases where plant DNA sequences are used, DNA sequences are
25 preferably used which code for a protein having one of the amino acid sequences given in Seq ID No. 1 or Seq ID No. 2 or Seq ID No. 3 or an essentially identical amino acid sequence. Shorter DNA sequences can also be used which code only for parts of the amino acid sequences given in Seq ID No. 1 Seq ID
30 No. 2 or Seq ID No. 3, provided that the resulting protein is guaranteed to have the enzymatic activity of a citrate synthase.

A particularly preferred embodiment consists of a process in which the DNA sequence coding for a citrate synthase activity
35 comprises the nucleotide sequence given in Seq ID No. 1 or Seq ID No. 2 or Seq ID No. 3, or an essentially identical



nucleotide sequence or a part thereof, this part being long enough to code for a protein which displays citrate synthase activity.

- 5 In addition, with the help of standard processes using DNA the already known sequences which code for citrate synthases, other DNA sequences can be isolated from any organisms, preferably from plants and prokaryotic organisms, which code for proteins having the enzymatic activity of a citrate synthase. These
10 sequences can also be used in the processes according to the invention.

- Using the process according to the invention, the citrate synthase activity can in principle be increased in every
15 compartment of a transformed cell. There will preferably be an increase in the activity in the mitochondria, the glyoxysomes or the cytosol. In order to guarantee localisation of the citrate synthase in a certain compartment of the transformed cells, the coding sequence must be linked to the sequences
20 necessary for localisation into the corresponding compartment. Such sequences are known. For localising the citrate synthase in the mitochondria it is for example necessary that the expressed protein has at the N-terminus a mitochondrial targeting sequence (signal sequence) which guarantees the
25 transportation of the protein expressed in the cytosol into the mitochondria. If the gene used does not already comprise a sequence which codes for a signal peptide, such a sequence must be introduced using genetic engineering methods. A sequence which codes for a mitochondrial targeting sequence is for
30 example known from Braun et al. (1992, EMBO J. 11: 3219-3227). The sequence must be linked to the coding region in such a way that the polypeptide coded by the target sequence lies in the same reading frame as the subsequent DNA sequence coding for citrate synthase.
- 35 If bacterial DNA sequences are used which code for a citrate synthase, then all 5'-non-translated regions are preferably



removed in these. If the bacterial enzyme has signal sequences, then these are preferably replaced by plant signal sequences.

5 The same as was already stated above in connection with the processes according to the invention for inhibiting flower formation applies to the choice of suitable transcriptional regulatory sequences, in particular promoters for expressing the DNA sequence which codes for citrate synthase and termination signals.

10

The described process can be used both on dicotyledons and on monocotyledons. Plants which are of particular interest are useful plants such as types of grain (e.g. rye, wheat, corn, barley, maize etc.), types of fruit (e.g. apricots, peaches, 15 apples, plums etc.), types of vegetables (e.g. tomatoes, paprika, pumpkin, melons, gherkins, courgettes, broccoli, asparagus etc.), ornamental plants or other economically interesting types of plants (e.g. tobacco, rapeseed, soya beans, cotton, sunflowers etc.).

20

A subject of the invention are also the plants obtainable from the described process which are characterized in that they display an increased citrate synthase activity in the cells because of the additional expression of a DNA sequence which 25 codes for a protein having the enzymatic activity of a citrate synthase. Such plants are also characterized in that they contain an expression cassette stably integrated into the genome, which comprises the following sequences:

30

A) a promoter functional in plants,

B) a DNA sequence coding for citrate synthase which is fused to the promoter in *sense* orientation, and if necessary

35

C) a signal functional in plants for the transcription termination and polyadenylation of an RNA molecule.



The plants are preferably those listed above.

By combining the DNA sequences according to the invention in the described processes for inhibiting or for inducing flower
5 formation with exogenously regulatable control elements for the transcription, e.g. temperature-induced promoters, there also exists the possibility of chronologically determined flowering induction or flowering inhibition, depending on whether the DNA sequence is fused to the promoter in *sense* or *anti-sense*
10 orientation. Thus, promoters are known *inter alia* for a specific expression in flower buds (Huisjer et al. (192) EMBO J. 11:1239-1249) or in photosynthetically active tissues, e.g. the ST-LS1 promoter (Stockhaus et al., 1989, EMBO J.8:2445-2451). To prevent the sprouting of potato tubers, and the
15 storage losses through metabolization of the storage substances, appropriate promoters are those which ensure an activation of the transcription in the storage organs. In the case of potatoes, promoters are known which ensure an expression specifically in the tuber, e.g. promoters of class
20 I patatin genes. An example is the promoter of the patatin gene B33 of *Solanum tuberosum* (Rocha-Sosa et al., 1989, EMBO J. 8:23-29). Through combination with exogenously regulatable control elements, for example wound-inducible or temperature-regulated promoters, the problem of vegetative multiplication
25 in the case of potato plants whose tubers do not sprout upon inhibition of the citrate synthase can be solved.

In the case of sugar beet, in analogous manner by using a beet-specific promoter, respiration can be reduced and consequently a yield loss through sugar degradation in the beet can be
30 lessened.

For preparing the introduction of foreign genes into higher plants, a large number of cloning vectors are available which contain a replication signal for *E. coli* and a marker gene for
35 the selection of transformed bacterial cells. Examples of such vectors are pBR322, pUC series, M13mp series, pACYC184 etc. The



desired sequence can be introduced into the vector at a suitable restriction cleavage site. The plasmid obtained is used for the transformation of *E. coli* cells. Transformed *E. coli* cells are grown in a suitable medium, then harvested and
5 lysed. The plasmid is recovered. Restriction analyses, gel electrophoreses and other biochemical-molecular biology methods are generally used as analysis method to characterize the plasmid DNA obtained. After each manipulation, the plasmid DNA can be cleaved and joined to other DNA sequences. Each plasmid
10 DNA sequence can be cloned in the same or other plasmids. A multitude of techniques are available for the introduction of DNA into a plant host cell. These techniques include the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation
15 agents, the fusion of protoplasts, injection, the electroporation of DNA, the introduction of DNA using the bio-ballistic method and other possibilities. For the injection and electroporation of DNA into plant cells, no special requirements as such are placed on the plasmids
20 used. Simple plasmids such as e.g. pUC derivatives can be used. If, however, whole plants are to be regenerated from cells transformed in this manner, the presence of a selectable marker gene is necessary. According to the method of introducing desired genes into the plant cell, other DNA sequences can be
25 necessary. If e.g. the Ti- or Ri-plasmid is used for the transformation of the plant cell, then at least the right border, although frequently the right and left border, of the Ti- and Ri-plasmid T-DNA must be joined as flanking region to the genes to be introduced.

30 If agrobacteria are used for the transformation, the DNA to be introduced must be cloned in special plasmids, either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti- or Ri-plasmid of the agrobacteria by homologous recombination because of sequences
35 which are homologous to sequences in the T-DNA. This also contains the *vir* region necessary for the transfer of the T-



DNA. Intermediate vectors cannot replicate in agrobacteria. By means of a helper plasmid, the intermediate vector can be transferred into *Agrobacterium tumefaciens* (conjugation). Binary vectors can replicate both in *E. coli* and in
5 agrobacteria. They contain a selection marker gene and a *linker* or *polylinker* which are framed by the right and left T-DNA border regions. They can be transformed directly into the agrobacteria (Holsters et al. (1978) Mol. Gen. Genet. 163:181-187). The agrobacterium serving as host cell has to contain a
10 plasmid which carries a *vir* region. The *vir* region is necessary for transferring the T-DNA into the plant cell. Additional T-DNA can be present. The agrobacterium transformed in this way is used for the transformation of plant cells.

The use of T-DNA for the transformation of plant cells has been
15 intensively investigated and adequately described in EP 120516; Hoekema, in: The Binary Plant Vector System Offsetdrukkerij Kanters B.V., Alblasterdam (1985), Chapter V; Fraley et al., Crit. Rev. Plant. Sci., 4: 1-46 and An et al. (1985) EMBO J. 4: 277-287.

20 To transfer the DNA into the plant cell, plant explants can be expediently co-cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Whole plants can then be regenerated from the infected plant material (e.g. pieces of leaves, stem
25 segments, roots or also protoplasts or suspension-cultivated plant cells) in a suitable medium which can contain antibiotics or biocides for the selection of transformed cells. The plants thus obtained can then be investigated for the presence of the introduced DNA.

30 Once the introduced DNA is integrated in the genome of the plant cell, it is as a rule stable there and is retained even in the successors of the cell originally transformed. It normally contains a selection marker which makes the
35 transformed plant cell resistant to a biocide or an antibiotic such as kanamycin, G 418, bleomycin, hygromycin or



phosphinothricin etc. The individually selected marker should therefore permit to distinguish transformed cells from cells which lack the introduced DNA.

- The transformed cells grow within the plant in the usual manner
- 5 (see also McCormick et al. (1986) Plant Cell Reports 5:81-84). The resulting plants can be grown normally and be crossed with plants which have the same transformed genetic code or other genetic codes. The hybrid individuals resulting therefrom have the appropriate phenotypic properties.
- 10 Two or more generations should be grown in order to ensure that the phenotypic feature is stably retained and inherited. Seeds should also be harvested in order to ensure that the corresponding phenotype or other characteristics are retained.
- 15 In addition to the uses already mentioned, the DNA sequences according to the invention can also be introduced into plasmids which permit a mutagenesis or a sequence modification through insertion, deletion or recombination of DNA sequences in prokaryotic or eukaryotic systems. The sequences can also be
- 20 provided with control elements for expression in pro- and eukaryotic cells and be introduced into the appropriate cells.

The DNA sequences according to the invention can also be used to isolate from the genome of plants of different species

25 homologous sequences which also code for a citrate synthase. In this context, homology means a sequence identity of at least 60 %, preferably above 80 % and in particular above 95 %. The identification and isolation of such sequences is carried out according to standard processes (see e.g. Sambrook et al.,

30 1989, Molecular Cloning, A Laboratory Manual, 2nd. ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY). With these sequences, constructions for the transformation of plants or microorganisms can in turn be produced.



Deposit

The plasmids produced and used within the scope of the present invention were deposited at the Deutsche Sammlung von
5 Mikroorganismen (German Collection of Microorganisms) (DSM) in Brunswick, Federal Republic of Germany, which is recognised as an international depository, in accordance with the requirements of the Budapest Treaty on the International
10 Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. On 28.12.1993 the following plasmids were deposited at the German Collection of Microorganisms (DSM) (Deposit number):

15 Plasmid pPCS (DSM 8879)

Plasmid pKS-CSa (DSM 8880)

On 10.08.1994 the following plasmids were deposited at the German Collection of Microorganisms (Deposit number):

20

Plasmid pTCS (DSM 9357)

Plasmid pSBCS (DSM 9358)

Plasmid TCSAS (DSM 9359)

25

Abbreviations used

| | |
|--------------------------|--|
| BSA | bovine serum albumin |
| EDTA | (ethylene dinitrilo) tetraacetic acid |
| 30 50x Denhardt solution | 5 g Ficoll (type 400, Pharmacia) |
| | 5 g polyvinyl pyrrolidone |
| | 5 g bovine serum albumin (Fraction V, Sigma) |
| | to 500 ml with H ₂ O |
| 35 FADH ₂ | flavin-adenine-dinucleotide, reduced |
| MOPS | 3-(N-morpholino)-propanesulphonic acid |



| | | |
|----|------------|---|
| | NADH | b-nicotinamide adenine dinucleotide, reduced |
| | PCR | polymerase chain reaction |
| | PMSF | phenyl methyl sulphonyl fluoride |
| 5 | SCMo-virus | "subterranean clover mottle virus" |
| | SDS | sodium dodecyl sulphate |
| | 20x SSC | 175.3 g NaCl, 88.2 g sodium citrate to 1000 ml with H ₂ O, pH 7.0 with 10 N NaOH |
| 10 | TobR-virus | "tobacco ringspot virus" |
| | Trizin | N-tris(hydroxymethyl) methyl glycine |

Description of the Figures

15

Fig. 1 shows the plasmid pPCS (DSM 8879)

20

The faint line corresponds to the sequence of pBluescript KS. The bold line represents the cDNA which codes for citrate synthase from *Solanum tuberosum*. Restriction cleavage sites of the insertion are shown.

25

Fig. 2 shows the plasmid pKS-CSa (DSM 8880)

30

Structure of the plasmid:

A= Fragment A: CaMV 35S promoter, nt 6909-7437 (Franck et al. (1980) Cell 21:285-294)

B= Fragment B: cDNA from *Solanum tuberosum* coding for citrate synthase;

BamHI/SalI-fragment from pPCS, approx. 1900 bp
Orientation to the promoter: *anti-sense*

C= Fragment C: nt 11748-11939 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846)

35



Fig. 3 shows the plasmid pSBCS (DSM 9358)

The faint line corresponds to the sequence of pBluescript SK. The bold line represents the cDNA which codes for citrate synthase from *Beta vulgaris* L. Restriction cleavage sites of the insertion are shown.

Fig. 4 shows the plasmid pTCS (DSM 9357)

The faint line corresponds to the sequence of pBluescript SK. The bold line represents the cDNA which codes for citrate synthase from *Nicotiana tabacum*. Restriction cleavage sites of the insertion are shown.

Fig. 5 shows the plasmid TCSAS (DSM 9359)

Structure of the plasmid:

A= Fragment A: CaMV 35S promoter, nt 6909-7437
(Franck et al. (1980) Cell 21:285-294)

B= Fragment B: cDNA from *Nicotiana tabacum*, coding for citrate synthase;
BamHI/SalI fragment from pTCS, approx. 1800 bp
Orientation to the promoter: anti-sense

C= Fragment C: nt 11748-11939 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846)

Fig. 6 shows the result of a Northern Blot experiment.

2 µg poly(A⁺)-mRNA from different transgenic potato plants (lanes 4-8) and three non-transformed potato plants (lanes 1-3) were used in each case for the analysis.

lanes 1, 2, and 3: Wild-type *Solanum tuberosum* cv. Désirée

lane 4: transgenic potato line T6



lane 5: transgenic potato line T21
lane 6: transgenic potato line T29
lane 7: transgenic potato line T50
lane 8: transgenic potato line T55

5

For the hybridization, the radioactively labelled cDNA of the citrate synthase from potatoes was used.

Fig. 7 shows transgenic potato plants of the line T6 (Nos. 3 and 4) and T29 (Nos. 5 and 6) which were transformed with the plasmid pKS-CSa, compared with wild-type plants (Nos. 1 and 2). The plants were kept in a greenhouse at 60 % humidity, at 22°C for 16 h in the light and at 15°C for 8 h in the dark.

15

Fig. 8 shows, as a diagram, the number of flowers produced in potato plants which had been transformed with the plasmid pKS-CSa, compared with wild-type plants. The number of plants with fully-developed open flowers during a flowering period is shown. 5 transgenic lines (T6, T21, T29, T50 and T55) are compared with wild-type plants. The transgenic line T21 is a transgenic line which displays no inhibition of the citrate synthase (100 % citrate synthase activity). During the period of investigation, no plant of the line T29 developed flowers and the plants of the lines T6 and T50 begin to flower only approx. 3 weeks later than wild-type plants. Day 1 stands for the first day on which clearly visible buds were to be seen on the plants.

wt = wild-type
t6, t21, t29, t50, t55 = transgenic lines T6, T21, T29, T50 and T55.

20

25

30

Fig. 9 shows longitudinal sections through flower buds of wild type plants and transgenic plants of the line

35



T29 for comparison

A: flower bud of a wild-type plant

B: Enlargement of the ovarian structure of the bud
from A

5 C: Flower bud of a plant of the transgenic line T29

D: Enlargement of the ovarian structure of the bud
from C

an: anthers

ov: ovary

10 pe: petals

se: sepals

The tissue damage in the ovaries of transgenic plants
is clearly visible.

15

Fig. 10 shows the germinating behaviour of tubers of potato
plants, of line T6 (left) which had been transformed
with the plasmid pKS-CSa, compared with tubers of
wild-type plants (right). The tubers had been stored
20 for 9 months in the dark at room temperature.

Fig. 11 shows a flower of a tobacco plant which had been
transformed with the plasmid TCSAS (left), compared
with a flower of a non-transformed tobacco plant
25 (right). The pistil of the flower of the transformed
plant is much shorter than the pistil of the flower
of the wild-type plant.

Fig. 12 shows the plasmid pHS-mCS
30 Structure of the plasmid:

A = Fragment A: CaMV 35S promoter, nt 6909-7437
(Franck et al. (1980) Cell 21:285-294)

B = Fragment B: 99 bp long DNA fragment which codes
for the mitochondria targeting sequence of the
35 matrix processing peptidase (MPP) (Braun et al.,
1992, EMBO J. 11:3219-3227)



C = Fragment C: DNA sequence from *Saccharomyces cerevisiae* coding for citrate synthase (nucleotides 376-1818; Suissa et al., 1984, EMBO J. 3:1773-1781)

5 orientation to the promoter: *sense*

D = Fragment D: nt 11748-11939 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846)

10 Fig. 13 shows two transgenic potato plants of two independent lines which had been transformed with the plasmid pHS-mCS (middle and right), compared with a wild-type plant (left). The plants were kept in a greenhouse and are approx. 6 weeks old. Whilst the wild type
15 plant has still formed no inflorescence, the two transgenic tobacco plants already have in each case a fully developed inflorescence.

Fig. 14 shows the plasmid pEC-mCS
20

Structure of the plasmid:

A = Fragment A: CaMV 35S promoter, nt 6909-7437 (Franck et al. (1980) Cell 21:285-294)

25 B = Fragment B: 99 bp long DNA fragment which codes for the mitochondria targeting sequence of the matrix processing peptidase (MPP) (Braun et al., 1992, EMBO J. 11:3219-3227)

30 C = Fragment C: DNA sequence from *E. coli* coding for citrate synthase (nucleotides 306-1589; Sarbjit et al., 1983, Biochemistry 22: 5244-5249)

orientation to the promoter: *sense*

35 D = Fragment D: nt 11748-11939 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846)



To provide a better understanding of the following examples, the most important processes used are explained below.

5 1. Cloning procedure

For the cloning in *E. coli* the vector pBluescriptKS and the vector pBluescriptSK (Stratagene, USA) were used.

- 10 For the plant transformation the gene constructions were cloned into the binary vector pBinAR.

2. Bacterial strains

- 15 For the pBluescript vectors and for the pBinAR vectors *E. coli* strain DH5 α (Bethesda Research Laboratories, Gaithersburg, USA) was used. For the *in vivo* excision the *E. coli* strain XL1-Blue was used.

- 20 The transformation of the plasmids into the potato plants and tobacco plants was carried out using the *Agrobacterium tumefaciens* strain C58C1 (Rocha-Sosa et al. (1989) EMBO J. 8:23-29).

25 3. Transformation of *Agrobacterium tumefaciens*

- The DNA was transferred by direct transformation according to the methods of Höfgen & Willmitzer (1988, Nucleic Acids Res. 16:9877). The plasmid DNA of transformed agrobacteria was
30 isolated according to the Birnboim & Doly method (1979, Nucleic Acid Res. 7:1513-1523) and analyzed by means of gel electrophoresis after suitable restriction cleavage.

4. Transformation of potatoes

35

Ten small scalpel-scored leaves of a potato sterile culture



(*Solanum tuberosum* L. cv. Désirée) were placed in 10 ml MS medium (Murashige & Skoog (1962) *Physiol. Plant.* 15: 473) with 2 % saccharose, which contained 50 µl of an *Agrobacterium tumefaciens* overnight culture, grown under selection. After 3-5 minutes' gentle shaking, they were further incubated for 2 days in the dark. After that, the leaves were placed on MS medium with 1.6 % glucose, 5 mg/l naphthyl acetic acid, 0.2 mg/l benzyl aminopurine, 250 mg/l Claforan, 50 mg/l kanamycin, and 0.80 % bactoagar for callus induction. After 1 week's incubation at 25°C and 3000 Lux the leaves were placed on MS medium with 1.6 % glucose, 1.4 mg/l zeatin ribose, 20 mg/l naphthyl acetic acid, 20 mg/l gibberellic acid, 250 mg/l Claforan, 50 mg/l kanamycin, and 0.80 % bactoagar for shoot induction.

15

5. Transformation of tobacco

An overnight culture of the corresponding *Agrobacterium tumefaciens* clone was centrifuged off (6500 rpm; 3 min) and the bacteria were resuspended in YEB medium. Tobacco leaves of a tobacco sterile culture (*Nicotiana tabacum* cv. Samsun NN) were cut into small approx. 1 cm²-sized pieces and bathed in the bacterial suspension. The leaf pieces were then placed on MS medium (0.7 % agar) and incubated for 2 days in the dark. The leaf pieces were then placed on MS medium (0.7 % agar) with 1.6 % glucose, 1 mg/l benzylaminopurine, 0.2 mg/l naphthyl acetic acid, 500 mg/l Claforan and 50 mg/l kanamycin for shoot induction. The medium was changed every 7 to 10 days. If shoots developed, the leaf pieces were transferred to glass vessels which contained the same medium. Forming shoots were cut off and placed on MS medium + 2 % saccharose + 250 mg/l Claforan and whole plants regenerated from them.

35



6. Determination of the citrate synthase activity in tissues of transgenic potato and tobacco plants and non-transformed potato and tobacco plants.

5 To determine the citrate synthase activity, raw extracts from tubers, leaves and flowers were produced and mitochondria isolated from potato tubers. To produce raw extracts, the material in question was frozen in liquid nitrogen, homogenized in extraction buffer (Neuhaus and Stitt (1990) Planta 182:445-
10 454), centrifuged, and the supernatant liquid was then used for the activity test. To isolate mitochondria from potato tubers, 100-200 g of freshly harvested tubers were peeled and homogenized in 100 ml "grinding buffer" (0.4 M mannitol, 1 mM EDTA, 25 mM MOPS, 0.1 % BSA, 10 mM b-mercaptoethanol, 0.05 mM
15 PMSF, pH 7.8). The homogenate was filtered through 4 layers of cotton gauze and centrifuged for 4 min at 3500 g. The supernatant was filtered through 2 layers of "Miracloth" (Calbiochem) and centrifuged again for 30 min at 18000 g. The pellet was resuspended using a soft brush in 2 ml resuspension
20 buffer (0.4 M mannitol, 20 mM Trizol, 2 mM EDTA, pH 7.2). After homogenizing twice in a "potter" homogenizer, the extract was coated onto a discontinuous Percoll gradient and centrifuged for 1 h at 72000 g. Mitochondria were removed from the 28%/45% interphase, washed and centrifuged twice for 15 min at 14500 g
25 in "washing buffer" (0.4 M mannitol, 5 mM MOPS, 0.1 % BSA, 0.2 mM PMSF, pH 7.5). The mitochondria were then resuspended in 100 µl resuspension buffer. To determine the citrate synthase activity 5 µl of the mitochondria suspension were taken up in 100 µl extraction buffer (Neuhaus and Stitt (1990) Planta
30 182:445-454).

The citrate synthase activity was determined by means of spectrophotometry at 412 nm and 30°C according to the Srere method (1967, Methods in Enzymology 13:3-22).



7. RNA extraction and Northern Blot experiments

RNA was isolated from frozen plant material as described in Logemann et al. (1987, Anal. Biochem. 163:21-26). The RNA was
5 denatured in 40 % formamide. The RNA was then separated by gel electrophoresis on formaldehyde/agarose gels, and after the gel run, blotted on nylon membrane (Hybond N; Amersham, UK). Hybridization with a radioactively-labelled DNA sample was carried out according to standard methods.

10

8. Plant maintenance

Potato plants (*Solanum tuberosum*) were kept in a green house at 60 % humidity and 22°C for 16 h in the light and at 15°C for 8
15 h in the dark. Tobacco plants (*Nicotiana tabacum*) were kept in the green house at 60 % humidity and 25°C for 14 h in the light and for 10 h at 20°C in the dark.

20

25

30

35



Examples**Example 1**

5 Cloning of a cDNA of the citrate synthase from potato

To identify a cDNA from potato which codes for citrate synthase, a DNA fragment of the already-known cDNA of citrate synthase from *Arabidopsis thaliana* (Unger et al. (1989) Plant
10 Mol. Biol. 13:411-418) was firstly amplified. For this, whole DNA was extracted from green plant tissue of *Arabidopsis thaliana* plants and poly(A⁺)-mRNA was prepared from this. This was then used for the preparation of cDNA. Using the oligodesoxynucleotides

15

5'-AAGTGGATCCATGGTGTTCCTCCGCAGCGTAT-3' (SeqID No. 4)

and

20

5'-CATAGGATCCTTAAGCAGATGAAGCTTTCTTA-3' (SeqID No. 5),

which are complementary to the 5'- or 3'-end of the coding region of the cDNA of the citrate synthase from *Arabidopsis thaliana* (Unger et al. (1989) Plant Mol. Biol. 13: 411-418), a
25 1438 bp-long DNA fragment which codes for the citrate synthase from *Arabidopsis thaliana* was isolated from this cDNA preparation by a "polymerase chain reaction" (PCR). The oligonucleotides used additionally introduce BamHI cleavage sites at both ends of the amplified DNA fragment. The DNA
30 fragment resulting from the PCR reaction was digested with BamHI and ligated into the plasmid PUC9.2 cleaved with BamHI. The cDNA insertion of this plasmid was later used as a heterologous sample for identifying a cDNA coding for citrate synthase from potato.

35

To produce a cDNA library, poly(A⁺)-mRNA was isolated from



leaves of potato plants. Starting from the poly(A⁺)-mRNA, cDNA was produced which was provided with EcoRI/NotI-linkers and with which a cDNA library was placed in the vector Lambda ZAP II (Stratagene, USA) (Kossmann et al. (1992) *Planta* 188:7-12).

5 250000 plaques of this cDNA library were investigated using the heterologous sample from *Arabidopsis thaliana* for DNA sequences which are homologous to this. For this, the plaques were transferred onto nitrocellulose filters and denatured by NaOH treatment. The filters were then neutralized and the DNA fixed

10 on the filters using heat treatment. The filters were pre-hybridized in 25 % formamide, 0.5 % BSA, 1% SDS, 5xSSC, 5x Denhardt solution, 40 mM sodium phosphate buffer pH 7.2 and 100 mg/ml salmon sperm DNA for 2 hours at 42°C. The filters were then hybridized overnight at 42°C in 25 % formamide, 0.5 % BSA,

15 1 % SDS, 5xSSC, 5x Denhardt solution, 40 mM sodium phosphate buffer pH 7.2 and 100 µg/ml salmon sperm DNA after adding the P³²-labelled cDNA coding for citrate synthase from *Arabidopsis thaliana*. The filters were washed for 30 min in 5xSSC, 0.5 % SDS at 42°C and for 20 min in 3xSSC, 0.5 % SDS at 42°C.

20 Phage clones of the cDNA library which hybridized with the cDNA used from *Arabidopsis thaliana* were further purified using standard processes. Using the *in vivo* excision method, *E. coli* clones which contain a double-stranded pBluescript plasmid with the corresponding cDNA insertion in the EcoRI cleavage site of

25 the polylinker were obtained from positive phage clones. After checking the size and the restriction pattern of the insertions, a suitable clone was subjected to a sequence analysis.

30 **Example 2**

Sequence analysis of the cDNA insertion of the plasmid pPCS (DSM 8879)

35 The plasmid pPCS (Fig. 1) was isolated from an *E. coli* clone obtained according to Example 1 and its cDNA insertion was



determined by standard procedures using the dideoxy method (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467). The insertion is 1891 bp long. The nucleotide sequence (SeqID No. 1) is given below.

5

Example 3

Construction of the plasmid pKS-CSa (DSM 8880) and transfer of the plasmid into potato plants.

10

An approx. 1.9 kb long DNA fragment which has the sequence (Seq ID No. 1) given below and which contains the cloning region for citrate synthase from potatoes was isolated from the plasmid pPCS through BamHI/SalI digest. This DNA fragment was cloned into the vector pBinAR (Höfgen and Willmitzer (1990) Plant Sci. 66:221-230) cleaved using BamHI/SalI. The vector pBinAR is a derivative of the binary vector Bin19 (Bevan (1984) Nucleic Acids Res. 12:8711-8721).

The resulting plasmid was called pKS-CSa and is shown in Fig. 2.

20

By inserting the cDNA fragment an expression cassette results which is constructed as follows from fragments A, B and C (Fig. 2):

25

Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The fragment comprises the nucleotides 6909 to 7437 of the CaMV (Franck et al. (1980) Cell 21:285-294).

30

Fragment B comprises the protein-coding region of the citrate synthase from potatoes. This was isolated as described above as BamHI/SalI fragment from pPCS and fused to the promoter in pBinAR in *anti-sense* orientation.

35

Fragment C (192 bp) contains the polyadenylation signal of gene



3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846).

The size of plasmid pKS-CSa is approx. 12.9 kb.

5

The vector pKS-CSa was transferred into potato plants using *Agrobacterium tumefaciens*-conveyed transformation. Intact plants were regenerated from the transformed cells. The result of the transformation was that transgenic potato plants showed to varying degree a reduction in the mRNA coding for the citrate synthase (see Fig. 6). 2 µg poly(A⁺)-mRNA were hybridized in a Northern Blot experiment with the probe for citrate synthase from potatoes. The transcript coding for citrate synthase which occurs in wild-type plants (lanes 1 to 3) is shorter than the transcript of the *anti-sense* expression cassette (see for example lane 6), from which it can be seen that the degree to which a reduction in the endogenous transcripts has occurred in the different transgenic plant varies.

20

Transgenic potato plants which show a reduction in the mRNA coding for the citrate synthase were investigated in different tissues for citrate synthase activity. The results of these investigations of leaves, tubers and mitochondria isolated from tubers are shown in the following table.

25

Table 1

Citrate synthase activity (in nmol/min/mg protein) in different organs of the plants and in mitochondria

30

| | Wild type | T55 | T50 | T6 | T29 |
|-----------|-----------|-----------|----------|----------|---------|
| Leaves | 55.6±25.0 | 32.7±25.0 | 15.1±8.7 | 15.0±7.7 | 3.2±1.2 |
| | 100 % | 58.8 % | 27.1% | 27.0% | 5.8% |
| 35 Tubers | 8.5±3.4 | 4.9±0.8 | 1.1±0.3 | 1.6 ±0.5 | 2.0±0.8 |



| | | | | | |
|-----------|----------|---------|--------|--------|---------|
| Mitochon- | 1788±492 | 450±120 | 265±45 | 260±50 | 193±118 |
| dria | 100% | 25.2% | 14.8% | 14.5% | 9.3% |

Wild type = *Solanum tuberosum* cv. Désirée,

5 T55, T50, T6, T29 = independent, transgenic potato lines

Reducing the citrate synthase activity has a considerable effect on flower formation in the transgenic plants, the markedness of which depends on the extent of inhibition of the
10 citrate synthase activity.

Transformed potato plants in which the citrate synthase activity is greatly reduced (see Table 1) are inhibited in their flower formation to a great extent or completely (see Fig. 7).

15 Plants in which the citrate synthase activity is only moderately reduced show delayed flower formation and produce fewer flowers or develop only flower buds which do not further develop to functional flowers, but die. This is shown in Fig. 8. Shown here are the number of plants with fully developed
20 open flowers during one flowering period. 5 transgenic lines (T6, T21, T29, T50 and T55) are compared with wild-type plants. The transgenic line T21 is a transgenic line which displays no inhibition of the citrate synthase (100 % citrate synthase activity). During the term of the investigation, no plant of
25 the line T29 developed flowers and the plants of the lines T6 and T50 begin to flower only approx. 3 weeks later than wild-type plants.

Other plants do develop flowers but these are not functional
30 since the female reproductive organs (ovaries) are severely damaged. In these plants the ovaries disintegrate in the course of development. This is shown in Fig. 9. This figure shows longitudinal sections through flower buds of wild-type plants and transgenic plants of the line T29 in comparison. The
35 tissues of the ovaries of transgenic plants are severely damaged compared with wild-type plants.



Using the present invention it is therefore also possible to produce plants according to the process according to the invention in which the citrate synthase activity is inhibited to varying degrees, so that from the transgenic plants can be
 5 chosen those which have the desired phenotype, for example a complete inhibition of flower formation, or flower formation whose onset, compared with non-transformed plants, is delayed, or which do develop buds from which, however, no functional flowers develop.

10

Reducing the citrate synthase activity also has a drastic effect on various properties of the tubers of the transformed potato plants. For example, tubers of transformed potato plants show lower storage losses after relatively long storage periods
 15 than tubers from non-transformed plants. This is expressed in a smaller loss of fresh or dry weight during the course of storage. The following table shows values for fresh and dry weights of tubers of transformed potato plants (line T6) and wild-type plants of the Désirée variety. The tubers were stored
 20 for 9 months at room temperature. The tuber weights are given in percentages, relative to the tuber fresh weights at the start of storage. The values are average values from 3 to 12 measurements with the standard deviation given. The values of the dry or fresh weights of the tubers of wild-type plants
 25 after 9 months' storage were taken as 100 %.

Table 2

| | Wild type | T6 |
|-----------------------|------------|------------|
| 30 Tuber fresh weight | 68.7 ± 2.6 | 77.2 ± 1.3 |
| [%] | 100 % | 112.4 % |
| Tuber dry weight | 18.7 ± 2.6 | 21.7 ± 0.5 |
| [%] | 100 % | 116 % |

35 Wild type = *Solanum tuberosum* cv. Désirée,
 T6 = transgenic potato lines



The tubers of transformed potato plants also show a changed sprouting behaviour. The sprouts of these tubers, compared with tubers of wild-type plants, are substantially smaller and have a substantially lower fresh and dry weight. The following table shows values for fresh and dry weights of sprouts of tubers of transformed potato plants (line T6) and wild-type plants of the Désirée variety. The sprouts originate from tubers which were stored in the dark for 9 months at room temperature. The sprout weights are given in each case in grams. The values are average values from 3 to 12 measurements with the standard deviation given.

15

Table 3

| | Wild type | T6 |
|-----------------------|-------------|-------------|
| Sprouts | 2.1 ± 0.6 | 1.3 ± 0.4 |
| 20 Fresh weight [g] | | |
| Sprouts | 0.31 ± 0.12 | 0.23 ± 0.06 |
| Dry weight [g] | | |

25 Wild type = *Solanum tuberosum* cv. Désirée,
T6 = transgenic potato line

The modified sprouting behaviour is also illustrated by Fig. 10. Shown in each case are 3 tubers of the transformed potato line T6 and three tubers of a wild-type plant of the Désirée variety. The tubers were stored in the dark for 9 months at room temperature. The tubers of the transformed plants (left) form substantially smaller and shorter sprouts compared with the wild-type tubers (right).

35



Example 4

Cloning of a cDNA coding for citrate synthase from tobacco
(*Nicotiana tabacum*)

5

For the identification of a cDNA from *Nicotiana tabacum* which codes for citrate synthase, a cDNA bank of leaf tissue from tobacco was prepared as described in example 1 for potato. 250000 plaques of this cDNA bank were screened using a
10 radioactive DNA probe for sequences which code for citrate synthase. The cDNA from *Solanum tuberosum* which codes for citrate synthase (1.4 kb NruI/HindII fragment from pPCS; see examples 1 and 2, and SeqID No. 1) was used as a probe. The identification and isolation of phage clones which hybridized
15 with the radioactive DNA probe used took place as described in Example 1 with the difference that the plaques were transferred onto nylon membranes and the following buffer was used for the pre-hybridization and the hybridization: 0.25 M sodium phosphate buffer pH 7.2, 10 mM EDTA, 7 % SDS, 10 mg BSA.
20 Using the *in vivo excision* method, *E. coli* clones were obtained from positive phage clones which contain a double-stranded pBluescript plasmid with the cDNA insertion in question. After checking the size and the restriction pattern of the insertions, a suitable clone was subjected to a sequence
25 analysis.

Example 5

Sequence analysis of the cDNA insertion of the plasmid pTCS
30 (DSM 9357)

The plasmid pTCS (Fig. 4) was isolated from an *E. coli* clone obtained according to Example 4 and its cDNA insertion was determined by standard procedures using the dideoxy method
35 (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467). The insertion is 1747 bp long. The nucleotide sequence



is given below as SeqID No. 3.

Example 6

- 5 Cloning of a cDNA coding for citrate synthase from sugar beet
(*Beta vulgaris* L.)

To identify a cDNA from sugar beet which codes for citrate
synthase, a cDNA bank of leaf tissue from sugar beet (*Beta*
10 *vulgaris* L. cultivated line 5S 0026) was prepared, isolating
poly(A⁺)-RNA from leaf tissue and using this for the cDNA
synthesis with the help of commercial kits (Pharmacia LKB,
Stratagene, USA) according to the Gubler and Hoffmann method
(1983, Gene 25:263-269). 250000 plaques of such a cDNA bank
15 were screened as described in Example 4 using radioactive DNA
probes for sequences which code for citrate synthase. Used as
the probe was a mixture consisting of the radioactively
labelled cDNA from *Solanum tuberosum* which codes for citrate
synthase (see Examples 1, 2, and 4, and SeqID No. 1), and the
20 radioactively-labelled cDNA from *Nicotiana tabacum* which codes
for citrate synthase (see Examples 4 and 5, and SeqID No. 3).
Phage clones which hybridized with the radioactive DNA sample
used were identified and isolated as described in Example 1.
Using the *in vivo* excision method, *E. coli* clones were obtained
25 from positive phage clones which contain a double-stranded
pBluescript plasmid with the cDNA insertion in question. After
checking the size and the restriction pattern of the
insertions, a suitable clone was subjected to a sequence
analysis.

30

Example 7

Sequence analysis of the cDNA insertion of the plasmid pSBCS
(DSM 9358)

35

The plasmid pSBCS (Fig. 3) was isolated from an *E. coli* clone



obtained according to Example 6 and its cDNA insertion was determined by standard procedures using the dideoxy method (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA 74: 5463-5467). The insertion is 1551 bp long. The nucleotide sequence is given as SeqID NO. 2 below.

Example 8

Construction of the plasmid TCSAS (DSM 9359) and transfer of the plasmid into tobacco plants.

An approx. 1,800 kb-long DNA fragment, which has the sequence given below (SeqID No. 3) and which contains the coding region for citrate synthase from *Nicotiana tabacum*, was isolated from the plasmid pTCS by BamHI/SalI digest. This DNA fragment was cloned into the vector pBinAR cleaved with BamHI/SalI (Höfgen and Willmitzer (1990) Plant Sci. 66:221-230). The vector pBinAR is a derivative of the binary vector Bin19 (Bevan (1984) Nucleic Acids Res. 12:8711-8721). The resulting plasmid was called TCSAS and is shown in Fig. 5.

By inserting the cDNA fragment an expression cassette results which is constructed of the fragments A, B and C in the following way (Fig. 5):

25

Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The fragment comprises the nucleotides 6909 to 7437 of the CaMV (Franck et al. (1980) Cell 21:285-294).

30

Fragment B contains, in addition to flanking regions, the protein-coding region of the citrate synthase from *Nicotiana tabacum*. This was isolated as described above as BamHI/SalI fragment from pTCS and fused in *anti-sense* orientation to the promoter in pBinAR.

35



Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846).

5 The size of plasmid TCSAS is approx. 12.75 kb.

The plasmid was transferred into tobacco plants using agrobacteria-conveyed transformation as described above. Whole plants were regenerated from the transformed cells.

10 The success of the genetic modification of the plants is tested by analyzing the whole RNA for the disappearance of the endogenous mRNA which codes for citrate synthase. Transgenic tobacco plants were investigated for citrate synthase activity in different tissues. The results of these investigations
15 showed that, with the help of the process, tobacco plants can be produced in which the citrate synthase activity is reduced to varying degrees.

As in the case of potato plants, different lines can therefore also be obtained with tobacco which differ as regards the
20 extent of reduction in the citrate synthase activity.

Also, in the case of tobacco plants, transformed plants showed a modified flowering behaviour. It is of particular interest that lines can be produced which produce flowers in which the
25 pistil is severely shortened, compared with flowers of non-transformed plants. This is illustrated by Fig. 11 in which flowers of transformed and non-transformed tobacco plants are shown. This means that the inhibition of flower formation by reducing the citrate synthase activity both in tobacco and in
30 the potato primarily affects the female flowering organs. These lines also display the phenotype, that they form substantially fewer seeds compared with wild-type plants, the quantity of seeds being determined with reference to the total weight of seeds formed.

35



Example 9

Construction of the plasmid pHS-mCS and transfer of the plasmid into potato plants.

5

To construct the plasmid pHS-mCS, a DNA sequence which codes for the mitochondrial targeting sequence of the matrix processing peptidase (MPP) was firstly integrated into a pUC18 vector. This sequence was isolated by means of the polymerase chain reaction (PCR) from a pBluescript plasmid which contained the cDNA sequence of the MPP (Braun et al., 1992, EMBO J. 11:3219-3227) using the following oligonucleotides:

Oligo a: 5'-GATC GGT ACC ATG TAC AGA TGC GCA TCG TCT-3'
15 (SeqID No. 6)

and

Oligo a: 5'-GTAC GGA TCC CTT GGT TGC AAC AGC AGC TGA-3'
(SeqID No. 7)

20 The resulting DNA fragment comprised the nucleotides 299 to 397 of the sequence shown in Braun et al (1992, EMBO J. 11:3219-3227), which codes for the matrix processing peptidase. An Asp 718 cleavage site was inserted at the 5'-end of the sequence by oligonucleotide a. Oligonucleotide b inserted a BamHI cleavage
25 site at the 3'-end of the sequence.

The DNA fragment obtained from the PCR was cleaved with Asp718 and BamHI and cloned into the vector pUC18 cleaved with Asp718 and BamHI. The resulting vector was called pMTP.

30 A DNA sequence from *Saccharomyces cerevisiae* which codes for a citrate synthase was cloned into the plasmid pMTP behind the mitochondrial targeting sequence in the same reading frame,. For this, genomic DNA was prepared from yeast by current methods and a 1443 bp-long fragment which comprises the coding
35 region for citrate synthase from yeast was isolated by means of PCR using the oligonucleotides



Oligo c: 5'- CTAG GGA TCC ATG TCA GCG ATA TTA TCA ACA ACT AGC
AAA AGT-3' (SeqID No. 8)

and

Oligo d: 5'- GATT GGA TCC TTA GTT CTT ACT TTC GAT TTT CTT TAC
5 CAA CTC-3' (SeqID No. 9)

In particular, the sequence comprises the nucleotides 376-1818
of the sequence illustrated in Suissa et al. (1984, EMBO J.
3:1773-1781). The oligonucleotides used introduce a BamHI
10 cleavage site on both sides of the amplified DNA sequence. The
resulting DNA fragment was cleaved with the restriction
endonuclease BamHI, then ligated into the vector pMTP cleaved
with BamHI and transformed in *E. coli* cells. By determining the
restriction pattern a clone was selected in which the insertion
15 of the PCR fragment took place in such a way that the coding
region was joined to the mitochondrial targeting sequence in
sense orientation, i.e. such that the 5'-end of the coding
region was joined to the 3'-end of the targeting sequence. The
resulting plasmid was called pMTP-YCS.

20

Using the restriction endonucleases Asp718 and Xba I, an
approx. 1550 bp-long fragment which comprises the mitochondrial
targeting sequence and the coding region for citrate synthase
from yeast was isolated from the vector pMTP-YCS. This fragment
25 was ligated into the binary vector pBinAR (Höfgen and
Willmitzer, 1990, Plant Sci. 66: 221-230) cleaved with Asp718
and Xba I. The resulting plasmid pHS-mCS is shown in Fig. 12.
The binary vector pBinAR is a derivative of the binary vector
Bin19. The vector contains a 35S promoter and a termination
30 signal for the transcription, between which is located a
polylinker which can be used for inserting various DNA
sequences.

By inserting the DNA fragment which codes for citrate synthase
35 from yeast with a mitochondrial target sequence at the N-
terminus, an expression cassette results which is constructed



of fragments A, B and C in the following manner (Fig. 12):

Fragment A (529 bp) contains the 35 S promoter of the cauliflower mosaic virus (CaMV). The fragment comprises the
5 nucleotides 6909 to 7437 of the CaMV (Franck et al. (1980) Cell 21:285-294).

Fragment B contains a 99 bp-long DNA fragment which codes for the mitochondrial target sequence of the matrix processing
10 peptidase (nucleotides 299-397 of the sequence shown in Braun et al., 1992, EMBO J. 11:3219-3227).

Fragment C contains the coding region for citrate synthase from *Saccharomyces cerevisiae* (nucleotides 376-1818 of the sequence
15 shown in Suissa et al., 1984 EMBO J. 3:1773-1781) fused in sense orientation and in the same reading frame as the target sequence to the 3'-end of the target sequence.

Fragment D (192 bp) contains the polyadenylation signal of gene
20 3 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846).

The size of the plasmid pHS-mCS is approx. 12.5 kb.

25 From this expression cassette, a transcript is transcribed by the 35S promoter which codes for a citrate synthase from yeast and comprises at its N-terminus an amino acid sequence which ensures transportation of the protein into the mitochondria.

30 The plasmid was transferred into potato plants using agrobacteria-conveyed transformation as described above. Whole plants were regenerated from the transformed cells.

The result of the transformation was that transgenic potato plants showed an expression of the yeast citrate synthase in
35 the cells. This was demonstrated with the help of Western Blot analyses using polyclonal antibodies which specifically



recognise the citrate synthase from yeast.

The transformed potato plants which showed a high expression of the citrate synthase from yeast display a modified flowering behaviour compared with non-transformed potato plants. On the one hand it was to be observed that transformed plants start to produce flowers substantially earlier (under green house conditions, on average 2-4 weeks) and produced more flowers compared with non-transformed plants.

The premature flower formation of the transgenic potato plants is illustrated in Fig. 13. This shows two transgenic potato plants which had been transformed with plasmid pHS-mCS, compared with a wild type plant of the Désirée variety.

The transgenic plants also produced substantially more flowers. In particular, after the first inflorescence had faded, the transgenic plants as a rule developed a second inflorescence and in some cases even a third inflorescence. In contrast, wild-type plants have only one florescence and die when this inflorescence has faded.

20 **Example 10**

Construction of the plasmid pEC-mCS and transfer of the plasmid into potato plants.

25 To produce the plasmid pEC-mCS, a DNA sequence from *E. coli* which codes for a citrate synthase was cloned into the plasmid pMTP described in Example 9 behind the mitochondrial targeting sequence in the same reading frame. For this, genomic DNA was prepared from *E. coli* DH5 α by current methods and an approx. 30 1280 bp-long fragment which comprises the coding region for citrate synthase from *E. coli* was isolated by means of PCR using the oligonucleotides

Oligo e: 5'- GTAGGGATCC ATGGCTGATA CAAAAGCAA - 3'
35 (SeqID No. 10)



and

Oligo f: 5'- GATTGGATCCTTAACGCTTGATATCGCTT - 3'
(SeqID No. 11)

5 The sequence comprises in particular the nucleotides 306-1589
of the sequence illustrated in Sarbjit et al. (1983,
Biochemistry. 22:5243-5249). The oligonucleotides used
introduce a BamHI cleavage site at both sides of the amplified
DNA sequence. The resulting DNA fragment was cleaved with the
10 restriction endonuclease BamHI, then ligated into the vector
pMTP cleaved with BamHI and introduced into *E. coli* cells by
transformation. By determining the restriction pattern, a clone
was selected in which the insertion of the PCR fragment took
place in such a way that the coding region was joined to the
15 mitochondrial targeting sequence in sense orientation, i.e.
that the 5'-end of the coding region was joined to the 3'-end
of the targeting sequence. The resulting plasmid was called
pMTP-ECCS. Using restriction endonucleases Asp718 and Xba I a
fragment was isolated from this vector which comprises the
20 mitochondrial targeting sequence and the coding region for
citrate synthase from *E. coli*. This fragment was ligated into
the binary vector pBinAR cleaved with Asp718 and Xba I (Höfgen
and Willmitzer, 1990, Plant Sci. 66:221-230). The resulting
plasmid pEC-mCS is illustrated in Fig. 14.

25 By inserting the DNA fragment which codes for citrate synthase
from *E. coli* with a mitochondrial targeting sequence at the N-
terminus, an expression cassette results which is constructed
from the fragments A, B, C and D in the following manner (Fig.
30 14):

Fragment A (529 bp) contains the 35 S promoter of the
cauliflower mosaic virus (CaMV). The fragment comprises the
nucleotides 6909 to 7437 of the CaMV (Franck et al. (1980) Cell
35 21:285-294).



Fragment B contains a 99 bp-long DNA fragment which codes for the mitochondrial targeting sequence of the matrix processing peptidase (nucleotides 299-397 of the sequence shown in Braun et al., 1992, EMBO J. 11:3219-3227).

5

Fragment C contains the coding region for citrate synthase from *E. coli* (nucleotides 306-1589 of the sequence shown in Sarbjit et al., 1983, Biochemistry. 22:5243-5249) fused in sense orientation and in the same reading frame as the targeting sequence to the 3'-end of the targeting sequence.

10

Fragment D (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti plasmid pTiACH5 (Gielen et al. (1984) EMBO J. 3:835-846).

15

The size of the plasmid pEC-mCS is approx. 12.4 kb.

From this expression cassette, a transcript is transcribed by the 35S promoter which codes for a citrate synthase from *E. coli* and comprises at its N-terminus an amino acid sequence which ensures transportation of the protein into the mitochondria.

20

The plasmid was transferred into potato plants using agrobacteria-conveyed transformation as described above. Whole plants were regenerated from the transformed cells and analyzed for citrate synthase activity.

25



SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

(A) NAME: Hoechst Schering AgrEvo GmbH

(B) STREET: Miraustr. 54

(C) CITY: Berlin

10

(E) COUNTRY: Germany

(F) POSTAL CODE (ZIP): 13476

(G) TELEPHONE: +49 30 439080

(H) TELEFAX: +49 30 43908222

15

(ii) TITLE OF INVENTION: Processes for inhibiting and inducing flower
formation in plants

(iii) NUMBER OF SEQUENCES: 11

20

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: DE P4408629.6

(B) FILING DATE: 09-MAR-1994

30

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: DE P4435366.9

(B) FILING DATE: 22-SEP-1994

(vi) PRIOR APPLICATION DATA:

35

(A) APPLICATION NUMBER: DE P4438821.7

(B) FILING DATE: 19-OCT-1994

(2) INFORMATION FOR SEQ ID NO: 1:

40

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1891 base pairs

(B) TYPE: nucleic acid



- 53 -

(C) STRANDEDNESS: unknown

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

5

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

10

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Solanum tuberosum

(B) STRAIN: c.v. Desiree

(F) TISSUE TYPE: leaf

15

(vii) IMMEDIATE SOURCE:

(A) LIBRARY: cDNA library in pBluescriptKS

(B) CLONE: pCBS

(ix) FEATURE:

20

(A) NAME/KEY: CDS

(B) LOCATION: 73..1485

(D) OTHER INFORMATION: /EC_number= 4.1.3.7.

/product= "Citrate synthase"

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTTTTCGTTC CATCAGCCTA CTTGAGATGT ATTCCCACTG GTAAAAGTTA ATTTTTTTTGA 60

30 TTTTCGCGAG CA ATG GTG TTC TAC CGT AGC GTT TCG TTG CTG TCA AAG 108

Met Val Phe Tyr Arg Ser Val Ser Leu Leu Ser Lys

1

5

10

CTC CGC TCT CGA GCG GTC CAA CAG TCA AAT GTT AGC AAT TCT GTG CGC 156

35 Leu Arg Ser Arg Ala Val Gln Gln Ser Asn Val Ser Asn Ser Val Arg

15

20

25

TGG CTT CAA GTC CAA ACC TCT TCC GGT CTT GAT CTG CGT TCT GAG CTG 204

Trp Leu Gln Val Gln Thr Ser Ser Gly Leu Asp Leu Arg Ser Glu Leu

40

30

35

40

GTA CAA GAA TTG ATT CCT GAA CAA CAG GAT CGC CTG AAA AAG ATC AAG 252

Val Gln Glu Leu Ile Pro Glu Gln Gln Asp Arg Leu Lys Lys Ile Lys



| | 45 | 50 | 55 | 60 | |
|----|---|-----|-----|-----|-----|
| | TCA GAT ATG AAA GGT TCA ATT GGG AAC ATC ACA GTT GAT ATG GTT CTT | | | | 300 |
| 5 | Ser Asp Met Lys Gly Ser Ile Gly Asn Ile Thr Val Asp Met Val Leu | 65 | 70 | 75 | |
| | GGT GGA ATG AGA GGA ATG ACA GGA TTA CTG TGG AAA CCT CAT TAC CTT | | | | 348 |
| | Gly Gly Met Arg Gly Met Thr Gly Leu Leu Trp Lys Pro His Tyr Leu | 80 | 85 | 90 | |
| 10 | GAC CCT GAT GAG GGA ATT CGC TTC CGG GGG TTG TCT ATA CCT GAA TGC | | | | 396 |
| | Asp Pro Asp Glu Gly Ile Arg Phe Arg Gly Leu Ser Ile Pro Glu Cys | 95 | 100 | 105 | |
| 15 | CAA AAG GTA TTA CCT GCA GCA AAG CCT GGG GGT GAG CCC TTG CCT GAA | | | | 444 |
| | Gln Lys Val Leu Pro Ala Ala Lys Pro Gly Gly Glu Pro Leu Pro Glu | 110 | 115 | 120 | |
| | GGT CTT CTC TGG CTT CTT TTA ACA GGA AAG GTG CCA TCA AAA GAG CAA | | | | 492 |
| 20 | Gly Leu Leu Trp Leu Leu Leu Thr Gly Lys Val Pro Ser Lys Glu Gln | 125 | 130 | 135 | 140 |
| | GTG AAT TCA ATT GTC TCA GGA ATT GCA GAG TCG GGC ATC ATA TCC CTG | | | | 540 |
| 25 | Val Asn Ser Ile Val Ser Gly Ile Ala Glu Ser Gly Ile Ile Ser Leu | 145 | 150 | 155 | |
| | ATC ATC ATG TAT ACA ACT ATT GAT GCC TTA CCA GTC ACA GCT CAT CCA | | | | 588 |
| | Ile Ile Met Tyr Thr Thr Ile Asp Ala Leu Pro Val Thr Ala His Pro | 160 | 165 | 170 | |
| 30 | ATG ACC CAG TTT GCT ACT GGA GTC ATG GCT CTT CAG GTT CAA AGT GAA | | | | 636 |
| | Met Thr Gln Phe Ala Thr Gly Val Met Ala Leu Gln Val Gln Ser Glu | 175 | 180 | 185 | |
| 35 | TTT CAA AAG GCA TAC GAG AAA GGG ATT CAC AAA TCA AAG TAT TGG GAA | | | | 684 |
| | Phe Gln Lys Ala Tyr Glu Lys Gly Ile His Lys Ser Lys Tyr Trp Glu | 190 | 195 | 200 | |
| | CCA ACA TAT GAG GAT TCC ATG AAT CTG ATT GCT CAA GTT CCA CTT GTT | | | | 732 |
| 40 | Pro Thr Tyr Glu Asp Ser Met Asn Leu Ile Ala Gln Val Pro Leu Val | 205 | 210 | 215 | 220 |



- 55 -

| | | |
|----|---|------|
| | GCT GCT TAT GTT TAT CGC AGG ATG TAC AAG AAT GGT GAC ACT ATA CCT | 780 |
| | Ala Ala Tyr Val Tyr Arg Arg Met Tyr Lys Asn Gly Asp Thr Ile Pro | |
| | 225 230 235 | |
| 5 | AAG GAT GAA TCC CTG GAT TAT GGT GCA AAT TTT GCT CAC ATG CTT GGT | 828 |
| | Lys Asp Glu Ser Leu Asp Tyr Gly Ala Asn Phe Ala His Met Leu Gly | |
| | 240 245 250 | |
| 10 | TTC AGT AGC TCT GAA ATG CAT GAA CTT CTT ATG AGG CTC TAT GTA ACA | 876 |
| | Phe Ser Ser Ser Glu Met His Glu Leu Leu Met Arg Leu Tyr Val Thr | |
| | 255 260 265 | |
| 15 | ATA CAC AGT GAT CAT GAA GGT GGT AAT GTC AGT GCT CAC ACC GGT CAC | 924 |
| | Ile His Ser Asp His Glu Gly Gly Asn Val Ser Ala His Thr Gly His | |
| | 270 275 280 | |
| 20 | TTG GTT GCT AGT GCT TTG TCT GAT CCT TAC CTC TCC TTT GCT GCT GCT | 972 |
| | Leu Val Ala Ser Ala Leu Ser Asp Pro Tyr Leu Ser Phe Ala Ala Ala | |
| | 285 290 295 300 | |
| | TTG AAT GGT TTA GCC GGA CCA CTT CAT GGT TTA GCC AAT CAG GAA GTT | 1020 |
| | Leu Asn Gly Leu Ala Gly Pro Leu His Gly Leu Ala Asn Gln Glu Val | |
| | 305 310 315 | |
| 25 | TTG CTA TGG ATA AAA TCT GTT GTA GAA GAA TGT GGG GAG AAC ATT TCC | 1068 |
| | Leu Leu Trp Ile Lys Ser Val Val Glu Glu Cys Gly Glu Asn Ile Ser | |
| | 320 325 330 | |
| 30 | AAA GAG CAG TTG AAA GAC TAT GTT TGG AAA ACA TTG AAC AGT GGC AAG | 1116 |
| | Lys Glu Gln Leu Lys Asp Tyr Val Trp Lys Thr Leu Asn Ser Gly Lys | |
| | 335 340 345 | |
| 35 | GTT GTC CCT GGT TTT GGA CAT GGA GTT CTG CGA AAG ACT GTA CCA AGA | 1164 |
| | Val Val Pro Gly Phe Gly His Gly Val Leu Arg Lys Thr Val Pro Arg | |
| | 350 355 360 | |
| 40 | TAT ACA TGC CAG AGA GAG TTC GCT ATG AAG CAT TTG CCT GAA GAT CCA | 1212 |
| | Tyr Thr Cys Gln Arg Glu Phe Ala Met Lys His Leu Pro Glu Asp Pro | |
| | 365 370 375 380 | |
| | CTG TTT CAA CTG GTT TCA AAA CTC TAC GAA GTT TTC CTC CTG TTC TTA | 1260 |
| | Leu Phe Gln Leu Val Ser Lys Leu Tyr Glu Val Phe Leu Leu Phe Leu | |
| | 385 390 395 | |



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CAG AAC TTG GCA AAG TTA AAA CCT TGG CCA AAT GTT GAT GCC CAC AGT 1308
 Gln Asn Leu Ala Lys Leu Lys Pro Trp Pro Asn Val Asp Ala His Ser
 400 405 410

5 GGT GTG TTG TTG AAC TAT TAT GGT TTA ACT GAA GCA AGA TAT TAT ACG 1356
 Gly Val Leu Leu Asn Tyr Tyr Gly Leu Thr Glu Ala Arg Tyr Tyr Thr
 415 420 425

10 GTC CTC TTT GGC GTA TCA AGA GCT CTT GGC ATT TGC TCT CAG CTA ATT 1404
 Val Leu Phe Gly Val Ser Arg Ala Leu Gly Ile Cys Ser Gln Leu Ile
 430 435 440

15 TGG GAC CGA GCT CTT GGA TTG CCG CTA GAG AGG CCA AAG AGT GTC ACA 1452
 Trp Asp Arg Ala Leu Gly Leu Pro Leu Glu Arg Pro Lys Ser Val Thr
 445 450 455 460

20 ATG GAG TGG CTT GAG AAC CAG TGC AAG AAA GCA TGAATTGTTT GAAATCTCGC 1505
 Met Glu Trp Leu Glu Asn Gln Cys Lys Lys Ala
 465 470

GAGCATAAAA CACAATGTAT AATCTCTATG AATAATTGCT TGACAAAGCA CTCCTTTCTT 1565

GGGGGACAAG ATAGGTCGGC CCTTCAATGG GTTAACGAAC TTCAGTTCAA ACTTCACTGA 1625

25 ATTTGTGTGA ATTGTATGGT TTCTCGAGAC TTGTCCTGAA TTTTGAACCT AGTCTAGTGG 1685

ATTCATTTTT CTTCATTCCG AATTCCTCAC ACGCTGATCC AGCATGTAAA AATTAATAGG 1745

30 TCAATGCTAT TAATCGCGTT CTTGGTTGCC ATTAGACTTG TGAATGACTT CCTTTGCTGG 1805

AAAGTTAGTA ATCGGCTGAT TCACGCAATA AACTGCAATT GTGTAGTTTC TTAAATTTGC 1865

TAATTCTTAT TTGATGATAT TATGAA 1891

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- 40 (A) LENGTH: 1551 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: linear



- 57 -

(ii) MOLECULE TYPE: cDNA to mRNA

(vi) ORIGINAL SOURCE:

5 (A) ORGANISM: Beta vulgaris
 (B) STRAIN: Zuchtlinie 5S 0026
 (F) TISSUE TYPE: leaf

(vii) IMMEDIATE SOURCE:

10 (B) CLONE: pSBCS

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION:1..1313
 15 (D) OTHER INFORMATION:/EC_number= 4.1.3.7.
 /product= "citrate synthase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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| 20 | TCC TCT AAC CTT GAC CTT CGT TCA GAG TTA CAA GAA CTG ATT CCT GAA | 48 |
| | Ser Ser Asn Leu Asp Leu Arg Ser Glu Leu Gln Glu Leu Ile Pro Glu | |
| | 475 480 485 | |
| 25 | CAA CAG GAA CGA CTG AAG AAG ATA AAG AAA GAA TTT GGA AGT TTC CAG | 96 |
| | Gln Gln Glu Arg Leu Lys Lys Ile Lys Lys Glu Phe Gly Ser Phe Gln | |
| | 490 495 500 | |
| 30 | CTG GGG AAT ATC AAT GTT GAC ATG GTA TTG GGC GGA ATG AGA GGA ATG | 144 |
| | Leu Gly Asn Ile Asn Val Asp Met Val Leu Gly Gly Met Arg Gly Met | |
| | 505 510 515 | |
| 35 | ACT GGT TTA CTT TGG GAG ACT TCC TTA CTC GAC CCA GAA GAG GGT ATC | 192 |
| | Thr Gly Leu Leu Trp Glu Thr Ser Leu Leu Asp Pro Glu Glu Gly Ile | |
| | 520 525 530 535 | |
| 40 | CGG TTC AGG GGT TTT TCT ATA CCT GAA TGC CAG AAA CTT TTA CCC GCT | 240 |
| | Arg Phe Arg Gly Phe Ser Ile Pro Glu Cys Gln Lys Leu Leu Pro Ala | |
| | 540 545 550 | |
| 40 | GCA AGT GCT GGT GCA GAG CCA TTG CCT GAA GGT CTT CTT TGG CTT CTT | 288 |
| | Ala Ser Ala Gly Ala Glu Pro Leu Pro Glu Gly Leu Leu Trp Leu Leu | |
| | 555 560 565 | |



| | | |
|----|---|-----|
| | TTA ACC GGA AAG GTT CCT AGC AAA GAG CAA GTA GAT GCT CTA TCA GCA | 336 |
| | Leu Thr Gly Lys Val Pro Ser Lys Glu Gln Val Asp Ala Leu Ser Ala | |
| | 570 575 580 | |
| 5 | GAT TTA CGA AAA CGT GCT TCT ATC CCA GAC CAT GTG TAC AAA ACA ATT | 384 |
| | Asp Leu Arg Lys Arg Ala Ser Ile Pro Asp His Val Tyr Lys Thr Ile | |
| | 585 590 595 | |
| 10 | GAT GCT CTA CCT ATT ACG GCT CAT CCA ATG ACT CAG TTT TGC ACT GGT | 432 |
| | Asp Ala Leu Pro Ile Thr Ala His Pro Met Thr Gln Phe Cys Thr Gly | |
| | 600 605 610 615 | |
| 15 | GTT ATG GCC TTA CAG ACT CGA AGC GAA TTT CAG AAG GCA TAT GAG AAA | 480 |
| | Val Met Ala Leu Gln Thr Arg Ser Glu Phe Gln Lys Ala Tyr Glu Lys | |
| | 620 625 630 | |
| 20 | GGG ATC CAT AAG TCA AAG TTT TGG GAG CCA ACA TAT GAG GAC TGC CTT | 528 |
| | Gly Ile His Lys Ser Lys Phe Trp Glu Pro Thr Tyr Glu Asp Cys Leu | |
| | 635 640 645 | |
| | AGT TTG ATT GCT CAA GTT CCT GTT GTT GCA GCT TAT GTT TAT CGG AGG | 576 |
| | Ser Leu Ile Ala Gln Val Pro Val Val Ala Ala Tyr Val Tyr Arg Arg | |
| | 650 655 660 | |
| 25 | ATG TAT AAG AAT GGA CAA GTA ATA CCG CTG GAT GAC TCC CTT GAT TAT | 624 |
| | Met Tyr Lys Asn Gly Gln Val Ile Pro Leu Asp Asp Ser Leu Asp Tyr | |
| | 665 670 675 | |
| 30 | GGT GGA AAT TTC GCA CAC ATG TTG GGA TTT GAT AGC CCT CAG ATG CTT | 672 |
| | Gly Gly Asn Phe Ala His Met Leu Gly Phe Asp Ser Pro Gln Met Leu | |
| | 680 685 690 695 | |
| 35 | GAG CTG ATG CGC CTT TAT GTC ACA ATT CAC AGT GAT CAT GAG GGT GGA | 720 |
| | Glu Leu Met Arg Leu Tyr Val Thr Ile His Ser Asp His Glu Gly Gly | |
| | 700 705 710 | |
| 40 | AAT GTT AGT GCA CAC ACT GGC CAT TTG GTG GGT AGT CCA CTT TCA GAT | 768 |
| | Asn Val Ser Ala His Thr Gly His Leu Val Gly Ser Pro Leu Ser Asp | |
| | 715 720 725 | |
| | CCT TAT TTG TCA TTT GCA GCA GCA TTA AAT GGT TTG GCT GGG CCA CTC | 816 |
| | Pro Tyr Leu Ser Phe Ala Ala Ala Leu Asn Gly Leu Ala Gly Pro Leu | |
| | 730 735 740 | |



| | | |
|----|---|------|
| | CAT GGA TTA GCC AAC CAG GAA GTC CTG CTG TGG ATT AAA TCA GTT GTT | 864 |
| | His Gly Leu Ala Asn Gln Glu Val Leu Leu Trp Ile Lys Ser Val Val | |
| | 745 750 755 | |
| 5 | GAT GAA TGT GGA GAG AAC ATC TCG ACA GAG CAG TTG AAA GAT TAT GTT | 912 |
| | Asp Glu Cys Gly Glu Asn Ile Ser Thr Glu Gln Leu Lys Asp Tyr Val | |
| | 760 765 770 775 | |
| 10 | TGG AAG ACA CTA AAC AGT GGC AAG GTT GTA CCT GGA TTT GGT CTA GGA | 960 |
| | Trp Lys Thr Leu Asn Ser Gly Lys Val Val Pro Gly Phe Gly Leu Gly | |
| | 780 785 790 | |
| 15 | GTA TTG CGG AAG ACA GAT CCA AGA TAC ACA TGC CAA AGA GAA TTT GCG | 1008 |
| | Val Leu Arg Lys Thr Asp Pro Arg Tyr Thr Cys Gln Arg Glu Phe Ala | |
| | 795 800 805 | |
| 20 | TTG AAG CAC TTG CCT GAT GAC CCA TTT TTT CAA TTG GTG TCA AAG TTG | 1056 |
| | Leu Lys His Leu Pro Asp Asp Pro Phe Phe Gln Leu Val Ser Lys Leu | |
| | 810 815 820 | |
| 25 | TAT GAA GTG GTG CCT CCT ATT CTA TTA GAG CTT GGA AAG GTA AAG AAT | 1104 |
| | Tyr Glu Val Val Pro Pro Ile Leu Leu Glu Leu Gly Lys Val Lys Asn | |
| | 825 830 835 | |
| 30 | CCA TGG CCT AAT GTT GAT GCT CAT AGT GGA GTT TTG CTG AAC CAC TAT | 1152 |
| | Pro Trp Pro Asn Val Asp Ala His Ser Gly Val Leu Leu Asn His Tyr | |
| | 840 845 850 855 | |
| 35 | GGT TTG ACA GAA GCA AGA TAC TAT ACG GTT TTG TTT GGG GTA TCA AGG | 1200 |
| | Gly Leu Thr Glu Ala Arg Tyr Tyr Thr Val Leu Phe Gly Val Ser Arg | |
| | 860 865 870 | |
| 40 | AGT CTT GGA ATA TGC TCA CAG CTT ATA TGG GAC CGA GCT CTT GGC TTG | 1248 |
| | Ser Leu Gly Ile Cys Ser Gln Leu Ile Trp Asp Arg Ala Leu Gly Leu | |
| | 875 880 885 | |
| 45 | CCG CTA GAG AGG CCA AAG AGT GTC ACT ATG GAA TGG CTT GAA AAG TTT | 1296 |
| | Pro Leu Glu Arg Pro Lys Ser Val Thr Met Glu Trp Leu Glu Lys Phe | |
| | 890 895 900 | |
| 50 | TGT AAA AGA AGA GCA TA ACATTGATGA CATATCAACT CACTGTTGTT | 1343 |
| | Cys Lys Arg Arg Ala | |
| | 905 | |



- 60 -

CTTTGTCGAA TCTACAATAA TATAGTTTGA GGGACAAGAA AGAATTTTAT TTTCGGAGAT 1403
GAGATAAGCG AGGACTCAGA AACATAGTTT TCTTTGTCTC TTGCTGAGGT TTGCGTTTAA 1463
5 TATATTTTAC TTGTAAATAT ATTGTATGGT TTCTTGATCA AAACATGAGA TAAAGAGTTT 1523
TCATAAAAAA AAAAAAAAAA AAAAAAAAAA 1551

10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
15 (A) LENGTH: 1747 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: linear
20 (ii) MOLECULE TYPE: cDNA to mRNA
(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Nicotiana tabacum*
(F) TISSUE TYPE: leaf
25 (vii) IMMEDIATE SOURCE:
(B) CLONE: TCS
(ix) FEATURE:
30 (A) NAME/KEY: CDS
(B) LOCATION: 70..1476
(D) OTHER INFORMATION: /EC_number= 4.1.3.7.
/product= "citrate synthase"

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCTCTTGGA TCTATTCCT CTCTCTATTT CTCCTAGGT AAAAGTTAAT TTGTTGATTT 60
40 TTGCGAGCC ATG GTG TTC TAT CGC GGC GTT TCT CTG CTG TCA AAG CTG 108
Met Val Phe Tyr Arg Gly Val Ser Leu Leu Ser Lys Leu
440 445 450



| | | |
|----|---|-----|
| | CGT TCT CGA GCG GTC CAA CAG ACA AAT CTT AGC AAC TCT GTG CGG TGG | 156 |
| | Arg Ser Arg Ala Val Gln Gln Thr Asn Leu Ser Asn Ser Val Arg Trp | |
| | 455 460 465 | |
| 5 | CTT CAA GTC CAA ACC TCT TCT GGT CTT GAT CTG CGT TCT GAG CTG CAA | 204 |
| | Leu Gln Val Gln Thr Ser Ser Gly Leu Asp Leu Arg Ser Glu Leu Gln | |
| | 470 475 480 | |
| | GAA TTG ATT CCA GAA CAA CAG GAT CGC CTA AAG AAG CTC AAG TCA GAG | 252 |
| 10 | Glu Leu Ile Pro Glu Gln Gln Asp Arg Leu Lys Lys Leu Lys Ser Glu | |
| | 485 490 495 | |
| | CAT GGA AAG GTT CAA TTG GGA AAC ATC ACA GTT GAT ATG GTT CTT GGT | 300 |
| 15 | His Gly Lys Val Gln Leu Gly Asn Ile Thr Val Asp Met Val Leu Gly | |
| | 500 505 510 | |
| | GGA ATG AGA GGA ATG ACA GGA TTA CTG TGG GAA ACC TCA TTA CTT GAC | 348 |
| | Gly Met Arg Gly Met Thr Gly Leu Leu Trp Glu Thr Ser Leu Leu Asp | |
| | 515 520 525 530 | |
| 20 | CCC GAT GAA GGA ATT CGC TTT CGG GGC TTG TCT ATC TAT GAA TGC CAA | 396 |
| | Pro Asp Glu Gly Ile Arg Phe Arg Gly Leu Ser Ile Tyr Glu Cys Gln | |
| | 535 540 545 | |
| | AAG GTA TTA CCT GCA GCA AAG CCT GGG GGA GAG CCC TTG CCT GAA GGT | 444 |
| 25 | Lys Val Leu Pro Ala Ala Lys Pro Gly Gly Glu Pro Leu Pro Glu Gly | |
| | 550 555 560 | |
| | CTT CTC TGG CTT CTT TTA ACA GGA AAG GTG CCA TCA AAA GAG CAA GTG | 492 |
| 30 | Leu Leu Trp Leu Leu Leu Thr Gly Lys Val Pro Ser Lys Glu Gln Val | |
| | 565 570 575 | |
| | GAT TCA TTG TCT CAG GAA TTG CGA AGT CGT GCT ACT GTC CCC GAT CAT | 540 |
| 35 | Asp Ser Leu Ser Gln Glu Leu Arg Ser Arg Ala Thr Val Pro Asp His | |
| | 580 585 590 | |
| | GTA TAC AAA ACT ATT GAT GCC TTA CCA GTC ACA GCT CAT CCA ATG ACT | 588 |
| | Val Tyr Lys Thr Ile Asp Ala Leu Pro Val Thr Ala His Pro Met Thr | |
| | 595 600 605 610 | |
| 40 | CAG TTT GCT ACT GGA GTC ATG GCT CTT CAG GTT CAA AGT GAA TTT CAA | 636 |
| | Gln Phe Ala Thr Gly Val Met Ala Leu Gln Val Gln Ser Glu Phe Gln | |
| | 615 620 625 | |



| | | |
|----|---|------|
| | AAG GCA TAT GAG AAA GGG ATT CAC AAA TCA AAG TTA TGG GAA CCG ACA | 684 |
| | Lys Ala Tyr Glu Lys Gly Ile His Lys Ser Lys Leu Trp Glu Pro Thr | |
| | 630 635 640 | |
| 5 | TAT GAG GAT TCC ATG AGT TTG ATT GCT CAA GTT CCA CTT GTT GCT GCT | 732 |
| | Tyr Glu Asp Ser Met Ser Leu Ile Ala Gln Val Pro Leu Val Ala Ala | |
| | 645 650 655 | |
| 10 | TAT GTT TAT CGC AGG ATG TAC AAG AAC GGC AAC ACT ATA CCT AAG GAT | 780 |
| | Tyr Val Tyr Arg Arg Met Tyr Lys Asn Gly Asn Thr Ile Pro Lys Asp | |
| | 660 665 670 | |
| 15 | GAC TCA CTG GAT TAT GGT GCA AAT TTT GCT CAC ATG CTT GGT TTC AGT | 828 |
| | Asp Ser Leu Asp Tyr Gly Ala Asn Phe Ala His Met Leu Gly Phe Ser | |
| | 675 680 685 690 | |
| 20 | AGC TCT GAC ATG CAT GAG CTT ATG AAG CTC TAT GTC ACG ATA CAC AGT | 876 |
| | Ser Ser Asp Met His Glu Leu Met Lys Leu Tyr Val Thr Ile His Ser | |
| | 695 700 705 | |
| | GAT CAT GAA GGT GGT AAC GTC AGT GCT CAC ACA GGT CAC TTG GTT GCT | 924 |
| | Asp His Glu Gly Gly Asn Val Ser Ala His Thr Gly His Leu Val Ala | |
| | 710 715 720 | |
| 25 | AGT GCT TTG TCA GAC CCT TAC CTC TCC TTC GCT GCT GCT TTG AAT GGT | 972 |
| | Ser Ala Leu Ser Asp Pro Tyr Leu Ser Phe Ala Ala Ala Leu Asn Gly | |
| | 725 730 735 | |
| 30 | TTA GCT GGA CCA CTT CAT GGT TTA GCC AAT CAG GAA GTT TTG CTA TGG | 1020 |
| | Leu Ala Gly Pro Leu His Gly Leu Ala Asn Gln Glu Val Leu Leu Trp | |
| | 740 745 750 | |
| 35 | ATC AAA TCT GTT GTA GAG GAG TGT GGG GAG AAC ATT TCC AAA GAG CAG | 1068 |
| | Ile Lys Ser Val Val Glu Glu Cys Gly Glu Asn Ile Ser Lys Glu Gln | |
| | 755 760 765 770 | |
| 40 | TTG AAA GAC TAC GCT TGG AAA ACA TTG AAA AGT GGC AAG GTT GTC CCT | 1116 |
| | Leu Lys Asp Tyr Ala Trp Lys Thr Leu Lys Ser Gly Lys Val Val Pro | |
| | 775 780 785 | |
| | GGT TTC GGA CAT GGA GTT CTG CGC AAG ACT GAT CCA AGA TAC ACA TGC | 1164 |
| | Gly Phe Gly His Gly Val Leu Arg Lys Thr Asp Pro Arg Tyr Thr Cys | |
| | 790 795 800 | |



| | | |
|----|--|------|
| | CAG AGA GAG TTC GCT TTG AAG CAT TTG CCT GAA GAT CCA CTG TTT CAA | 1212 |
| | Gln Arg Glu Phe Ala Leu Lys His Leu Pro Glu Asp Pro Leu Phe Gln | |
| | 805 810 815 | |
| 5 | CTG GTT GCA AAA CTC TAC GAA GTG TTC CTC CAA TTC TTA CAG AAC TTG | 1260 |
| | Leu Val Ala Lys Leu Tyr Glu Val Phe Leu Gln Phe Leu Gln Asn Leu | |
| | 820 825 830 | |
| 10 | GCA AAG TTA AAC CCT TGG CCA AAT GTT GAT GCC CAC AGT GGT GTG TTG | 1308 |
| | Ala Lys Leu Asn Pro Trp Pro Asn Val Asp Ala His Ser Gly Val Leu | |
| | 835 840 845 850 | |
| 15 | TTG AAC TAT TAT GGT TTA ACT GAA GCA AGA TAT TAT ACG GTC CTC TTT | 1356 |
| | Leu Asn Tyr Tyr Gly Leu Thr Glu Ala Arg Tyr Tyr Thr Val Leu Phe | |
| | 855 860 865 | |
| 20 | GGT GTA TCA AGA GCT CTT GGC ATT TGC TCT CAG CTA ATT TGG GAC CGA | 1404 |
| | Gly Val Ser Arg Ala Leu Gly Ile Cys Ser Gln Leu Ile Trp Asp Arg | |
| | 870 875 880 | |
| | GCT CTT GGA TTG CCA CTA GAG AGG CCA AAG AGT GTC ACA ATG GAG TGG | 1452 |
| | Ala Leu Gly Leu Pro Leu Glu Arg Pro Lys Ser Val Thr Met Glu Trp | |
| | 885 890 895 | |
| 25 | CTT GAG AAC CAT TGC AAG AAA GCA TGATTTGTTT GAAATCTCTG CGAGCATAAA | 1506 |
| | Leu Glu Asn His Cys Lys Lys Ala | |
| | 900 905 | |
| 30 | AGCACAATGT AAAATCTTTA TGAATAATTG CTTGAGAAAG CAGTTTTTTTC TTGGAGCCAA | 1566 |
| | GGTAGGTCGC ATTAGGATGT TCATCGATTG GCTTAGTACG GTTTTGAAAG ATTTTGTTG | 1626 |
| | TGTATTTTCA GTTTCGGTTT TAAAAATGTT ATACCAATAC CTTATCGATA TAAATTCAAT | 1686 |
| 35 | ATGATTCGAT TTTTACTTTT TGTTTGAAAA AAAAAACAAA AAAAAAAAAA AAAAAAAAAA | 1746 |
| | A | 1747 |
| 40 | | |

(2) INFORMATION FOR SEQ ID NO: 4:



- 64 -

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

10

(iii) HYPOTHETICAL: YES

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

AAGTGGATCC ATGGTGT TTTT TCCGAGCGT AT

32

20

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 32 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

30

(iii) HYPOTHETICAL: YES

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CATAGGATCC TTAAGCAGAT GAAGCTTTCT TA

32

40

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:



- 65 -

(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

15

GATCGGTACC ATGTACAGAT GCGCATCGTC T

31

(2) INFORMATION FOR SEQ ID NO: 7:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

30

(iii) HYPOTHETICAL: YES

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTACGGATCC CTTGGTTGCA ACAGCAGCTG A

31

40 (2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 base pairs



- 66 -

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

15 CTAGGGATCC ATGTCAGCGA TATTATCAAC AACTAGCAAA AGT

43

(2) INFORMATION FOR SEQ ID NO: 9:

20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "oligonucleotide"

30 (iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

35

GATTGGATCC TTAGTTCTTA CTTTCGATTT TCTTTACCAA CTC

43

(2) INFORMATION FOR SEQ ID NO: 10:

40

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid



- 67 -

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

5 (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTAGGGATCC ATGGCTGATA CAAAAGCAA

29

15

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

20

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

35 GATTGGATCC TTAACGCTTG ATATCGCTT

29



Claims

1. DNA sequences from a plant of the *Solanaceae* family or the
5 *Chenopodiaceae* family which contain the coding region for
a citrate synthase (EC No. 4.1.3.7.), characterized in
that the information contained in the nucleotide sequence
permits, upon integration into a plant genome, the
10 formation of transcripts through which an endogenous
citrate synthase activity can be suppressed, or permits
the formation of transcripts by which the citrate synthase
activity in the cells can be increased.
2. DNA sequences according to claim 1, characterized in that
15 these sequences originate from the species *Solanum*
tuberosum.
3. DNA sequences according to claim 1, characterized in that
these sequences originate from the species *Nicotiana*
tabacum.
20
4. DNA sequences according to claim 1, characterized in that
these sequences originate from the species sugar beet
(*Beta vulgaris*).
- 25 5. DNA sequences according to claim 1, characterized in that
these sequences code for a protein which has the amino
acid sequence given in SeqID No. 1 or an essentially
identical amino acid sequence.
- 30 6. DNA sequences according to claim 1, characterized in that
these sequences code for a protein which has the amino
acid sequence given in SeqID No. 2 or an essentially
identical amino acid sequence.
- 35 7. DNA sequences according to claim 1, characterized in that
these sequences code for a protein which has the amino



acid sequence given in SeqID No. 3 or an essentially identical amino acid sequence.

- 5 8. DNA sequences according to claim 1, characterized in that these sequences have the nucleotide sequence given in SeqID No. 1 or an essentially identical nucleotide sequence.
- 10 9. DNA sequences according to claim 1, characterized in that these sequences have the nucleotide sequence given in SeqID No. 3 or an essentially identical nucleotide sequence.
- 15 10. DNA sequences according to claim 1, characterized in that these sequences have the nucleotide sequence given in SeqID No. 2 or an essentially identical nucleotide sequence.
- 20 11. Process for inhibiting flower formation in plants, characterized in that the citrate synthase activity in the cells of the plants is reduced.
- 25 12. Process to improve the storage capability of storage organs in plants, characterized in that the citrate synthase activity in the cells of the plants is reduced.
- 30 13. Process for the production of transgenic tuberous plants, the tubers of which show reduced sprouting, characterized in that the citrate synthase activity in the cells of the plants is reduced.
- 35 14. Process according to one or more of claims 11 to 13, whereby the citrate synthase activity is reduced by inhibiting the expression of DNA sequences which code for citrate synthases.



15. Process according to claim 14, characterized in that the expression of DNA sequences which code for citrate synthases is inhibited by the use of *anti-sense* RNA.
- 5 16. Process according to claim 15, characterized in that
- a) a DNA which is complementary to a citrate synthase gene present in the cell is stably integrated into the genome of a plant cell,
 - 10 b) this DNA is expressed constitutively or is inducible due to the combination with suitable elements controlling the transcription,
 - c) the expression of endogenous citrate synthase genes is inhibited because of an *anti-sense* effect and
 - 15 d) plants are regenerated from the transgenic cells.
17. Process according to one or more of claims 15 to 16, wherein the DNA sequence transcribed into *anti-sense* RNA comprises a nucleotide sequence which codes in sense
- 20 orientation for a protein having the amino acid sequence given in SeqID No. 1 or SeqID No. 2 or SeqID No. 3 or an essentially identical amino acid sequence or a part thereof, whereby the coding sequence used is suitable for inhibiting the expression of an endogenous citrate
- 25 synthase gene.
18. Process according to one or more of claims 15 to 16, wherein the DNA sequence transcribed into *anti-sense* RNA
- 30 comprises the nucleotide sequence given in SeqID No. 1 or SeqID No. 2 or SeqID No. 3 or an essentially identical nucleotide sequence or a part thereof or derivatives thereof which are derived by insertion, deletion or
- 35 substitution of this sequence, whereby these parts or derivatives are suitable for inhibiting the expression of an endogenous citrate synthase gene.



19. Process for inducing flower formation in plants, characterized in that the citrate synthase activity in the cells of the plant is increased.
- 5
20. Process according to claim 19, characterized in that a recombinant DNA molecule is inserted into cells which comprises the coding region for a citrate synthase and which leads to the expression of a citrate synthase in the transformed cells.
- 10
21. Process according to claim 20, characterized in that
- 15
- a) DNA which is of homologous or heterologous origin and which codes for a protein having a citrate synthase activity is stably integrated into the genome of a plant cell,
 - b) this DNA is constitutively or inductively expressed by combining with suitable elements controlling the transcription,
 - c) because of this expression the citrate synthase activity in the transgenic cells increases and
 - d) plants are regenerated from the transgenic cells.
- 20
- 25
22. Process according to one or more of claims 20 to 21, wherein the DNA sequence coding for a citrate synthase codes for a deregulated or unregulated citrate synthase.
- 30
23. Process according to one or more of claims 20 to 21, wherein the DNA sequence coding for a citrate synthase comprises a nucleotide sequence which codes for a protein having the amino acid sequence given in SeqID No. 1 or SeqID No. 2 or SeqID No. 3 or an essentially identical amino acid sequence or for a part of these sequences
- 35



whereby these part displays citrate synthase activity.

24. Process according to one or more of claims 20 to 21,
5 wherein the DNA sequence coding for a citrate synthase
activity comprises the nucleotide sequence given in SeqID
No. 1 or Seq ID No. 2 or Seq ID No. 3 or an essentially
identical nucleotide sequence or a part thereof, whereby
10 this part is long enough to code for a protein which
displays citrate synthase activity.
25. Process according to one or more of claims 20 to 21
15 wherein the DNA sequence coding for a citrate synthase
originates from *Saccharomyces cerevisiae*.
26. Process according to one or more of claims 20 to 21,
20 wherein the DNA sequence coding for a citrate synthase
originates from a prokaryotic organism.
27. Process according to one or more of claims 20 to 21,
25 wherein the DNA sequence coding for a citrate synthase
originates from *E. coli*.
28. Recombinant double-stranded DNA molecules comprising an
expression cassette comprising the following constituents:
30
- i) a promoter functional in plants,
 - ii) a DNA sequence coding for citrate synthase which is
fused to the promoter in *anti-sense* orientation so
35 that the non-coding strand is transcribed, and if
necessary



iii) a signal functional in plants for the transcription termination and polyadenylation of an RNA molecule.

5 29. Recombinant double-stranded DNA molecules comprising an expression cassette comprising the following constituents:

A) a promoter functional in plants,

10 B) a DNA sequence coding for citrate synthase which is fused to the promoter in *sense* orientation, and if necessary

15 C) a signal functional in plants for the transcription termination and polyadenylation of an RNA molecule.

30. Plasmid pPCS which was deposited under DSM No. 8879.

20

31. Plasmid pKS-CSa which was deposited under DSM No. 8880.

32. Plasmid pSBCS which was deposited under DSM No. 9358.

25

33. Plasmid pTCS which was deposited under DSM No. 9357.

30 34. Plasmid TCSAS which was deposited under DSM No. 9359.

35. A plasmid, characterized in that it contains DNA sequences according to one or more of claims 1 to 10.

35



36. Bacteria, containing DNA sequences according to one or more of claims 1 to 10.
- 5 37. Bacteria containing DNA molecules according to claims 28 to 29.
- 10 38. Transgenic plants, containing DNA sequences according to one or more of claims 1 to 10 as a constituent of recombinant DNA.
- 15 39. Transgenic plants containing recombinant DNA molecules according to claims 28 to 29.
- 20 40. Transgenic plants, characterized in that they display reduced citrate synthase activity in the cells because of the expression of an *anti-sense* RNA which is complementary to DNA sequences which code for a protein having the enzymatic activity of citrate synthase.
- 25 41. Transgenic plants, characterized in that they show an increased citrate synthase activity in the cells because of the additional expression of a DNA sequence which codes for a protein having the enzymatic activity of citrate synthase.
- 30
42. Transgenic plant according to claims 38 to 41, characterized in that it is a useful plant.



43. Transgenic plant according to claims 38 to 42, characterized in that it is a potato.
- 5 44. Use of DNA sequences which code for citrate synthase (EC No. 4.1.3.7.), for inhibiting flower formation in plants.
- 10 45. Use of DNA sequences which code for citrate synthase (EC No. 4.1.3.7.) for inducing flower formation in plants.
- 15 46. Use of a DNA sequence according to one or more of claims 1 to 10 in combination with control elements for an expression in pro- and eukaryotic cells.
- 20 47. Use of a DNA sequence according to one or more of claims 1 to 10 for the expression of a non-translatable mRNA which prevents the synthesis of an endogenous citrate synthase in the cells.
- 25 48. Use of the DNA sequences according to claims 8, 9 or 10 for isolating homologous sequences from the genome of plants.



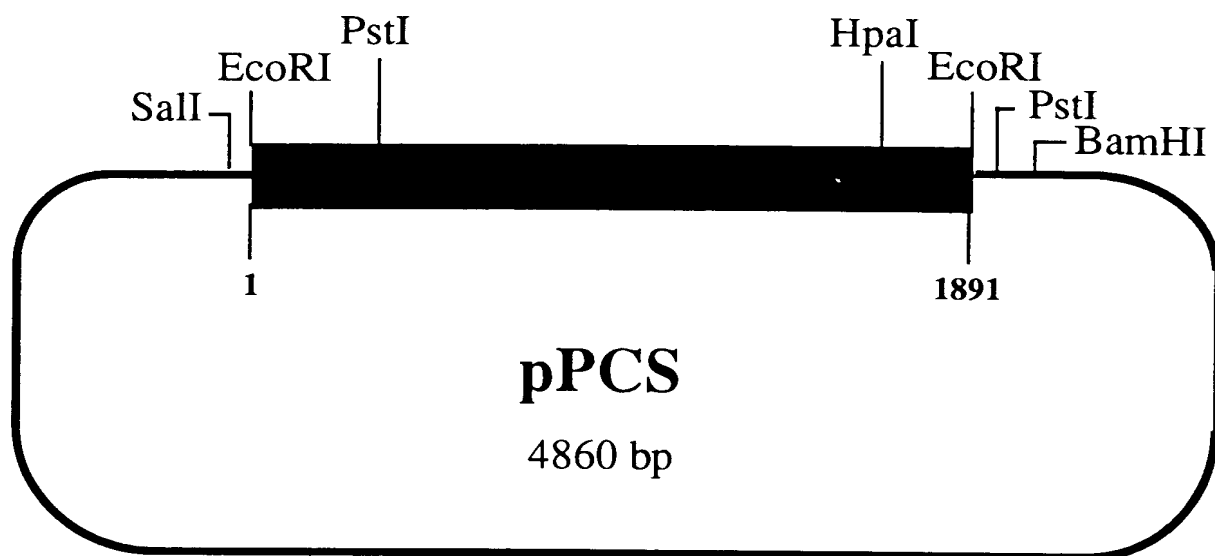


Fig. 1

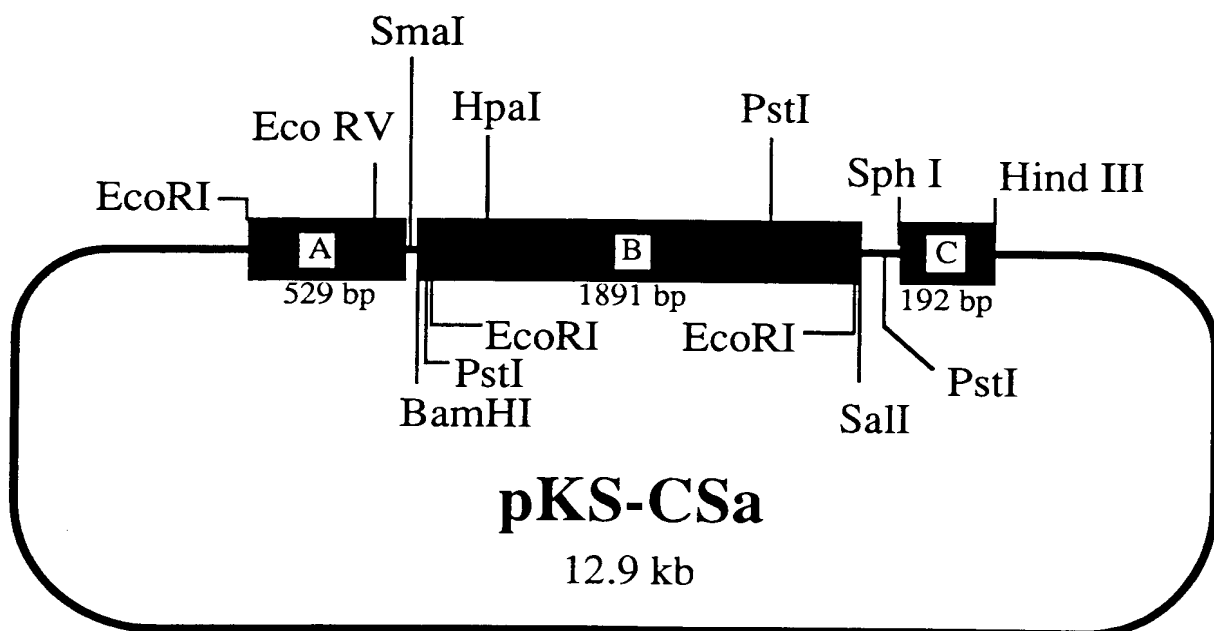


Fig. 2



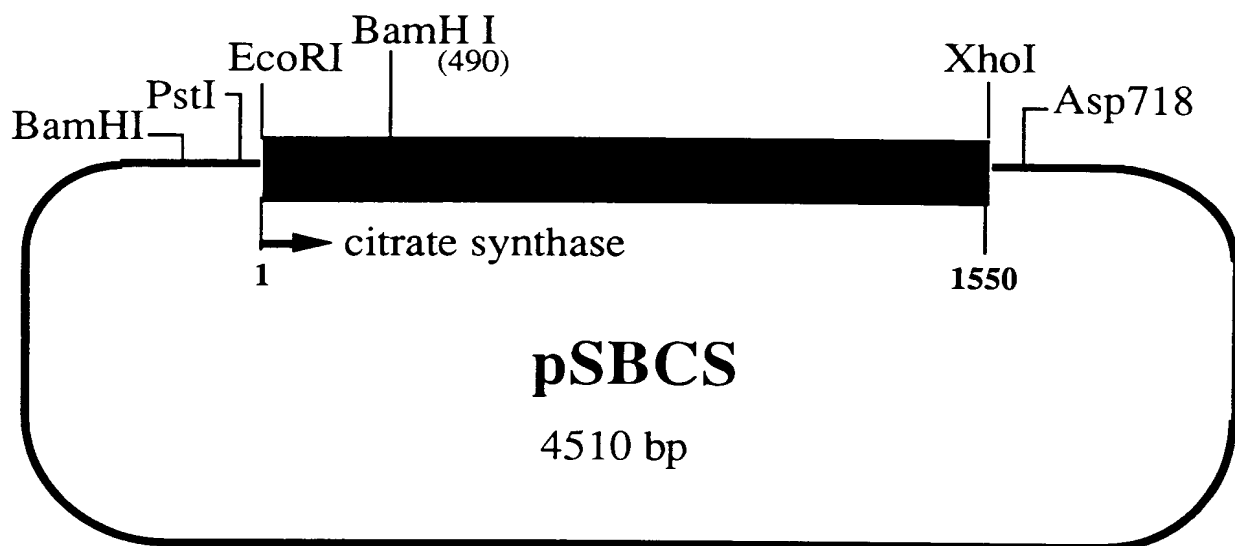


Fig. 3

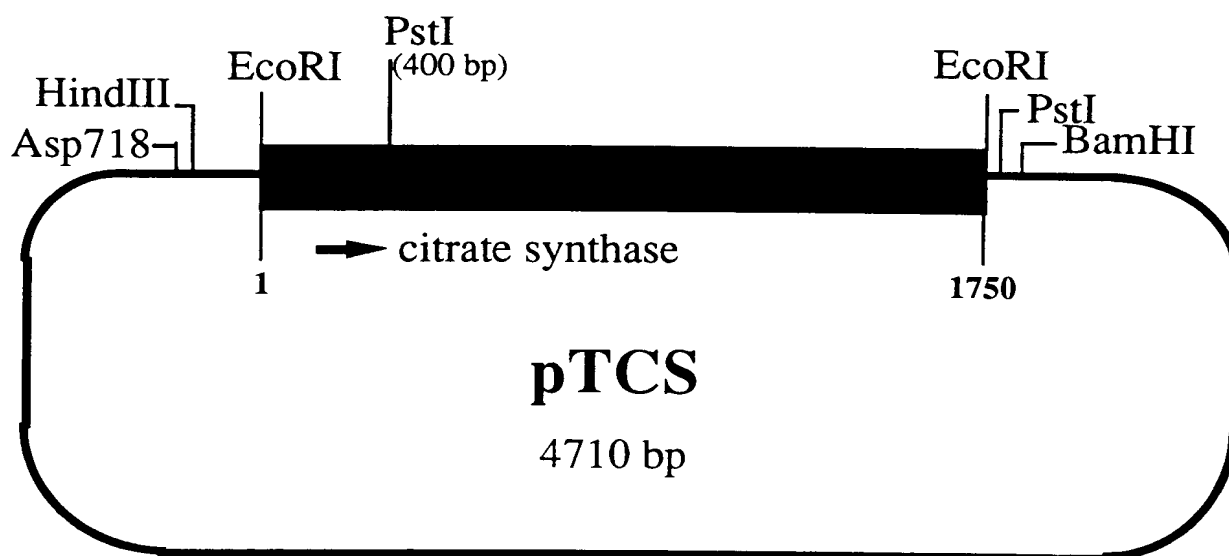


Fig. 4



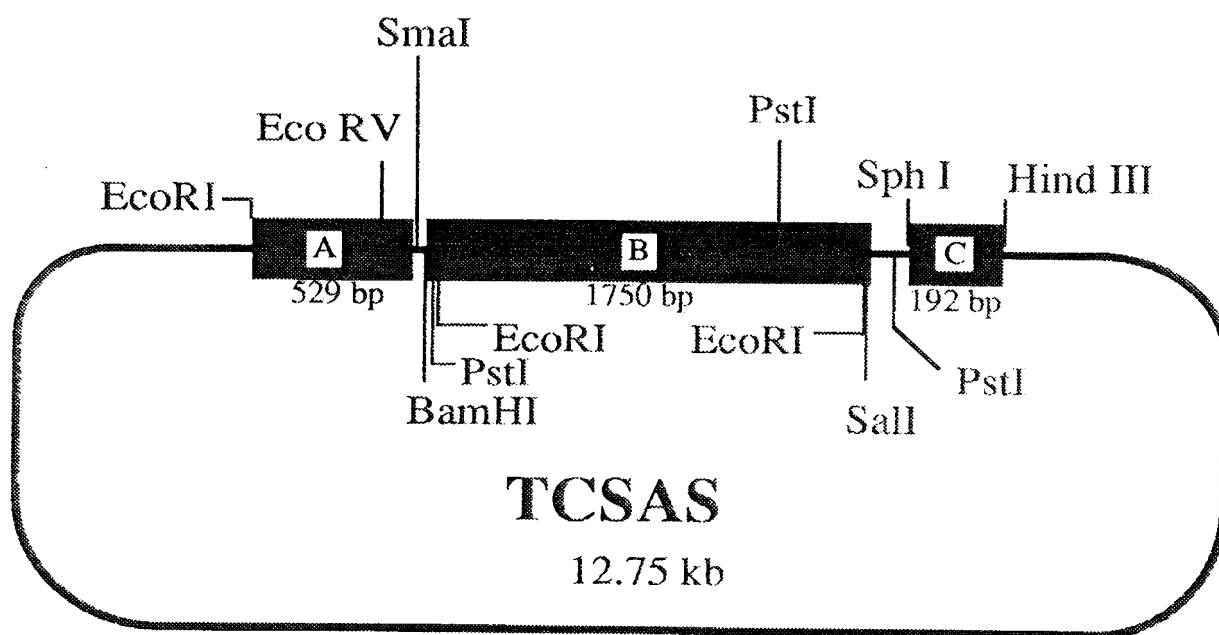


Fig. 5

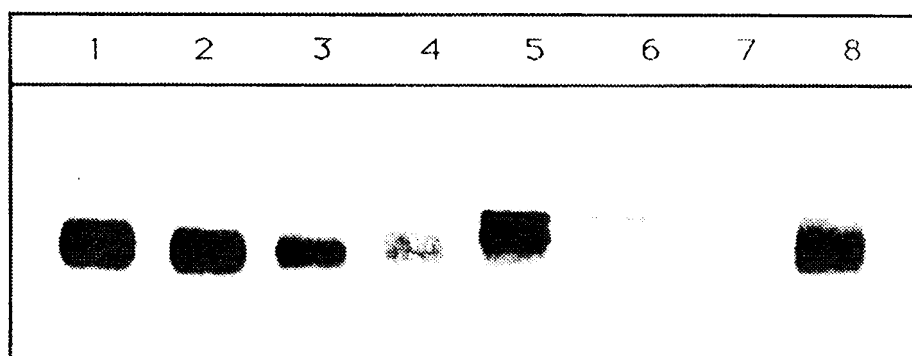


Fig. 6





Fig. 7

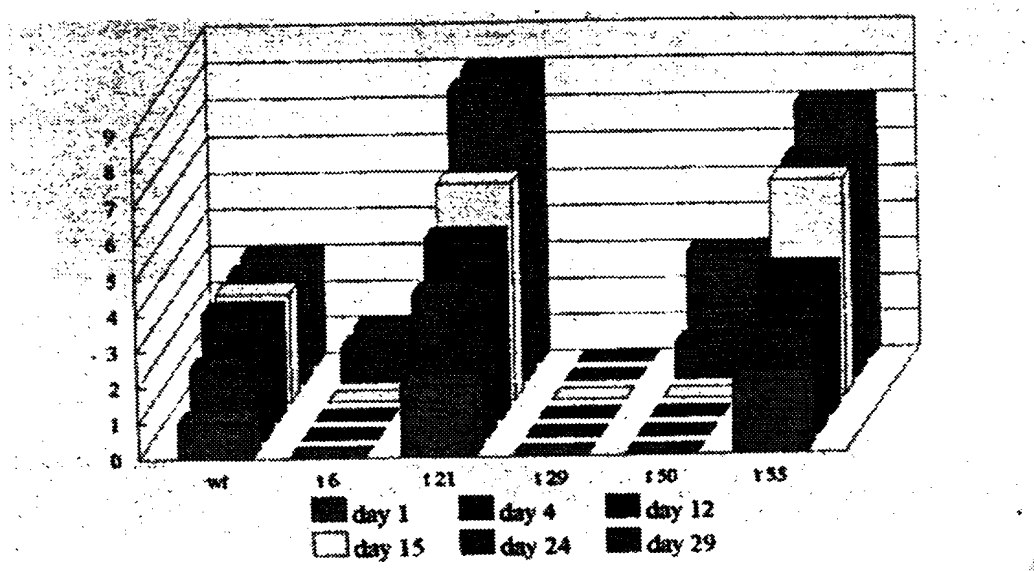


Fig. 8



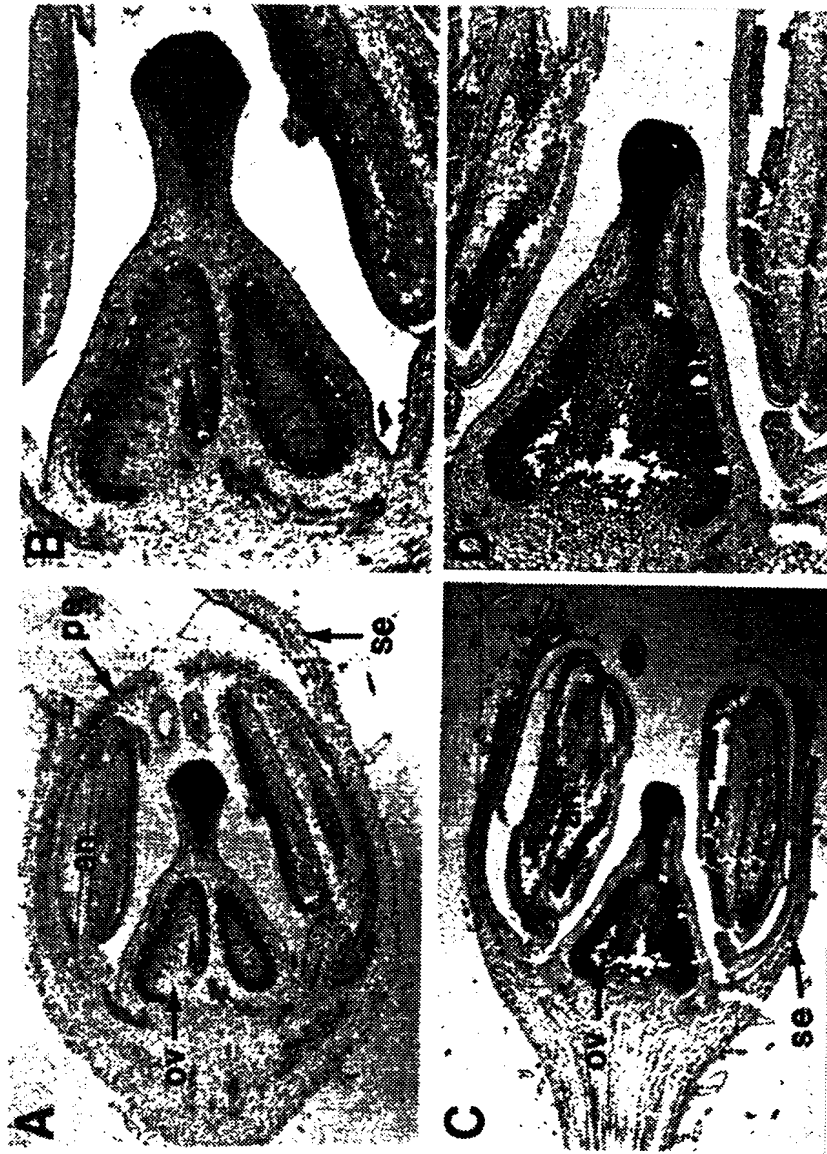


Fig. 9



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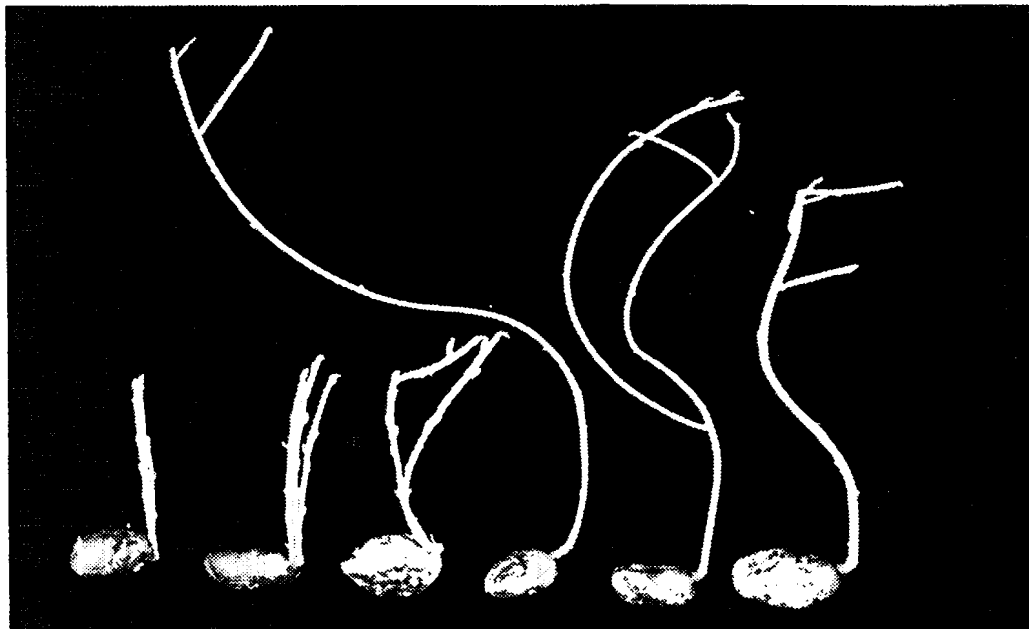


Fig. 10

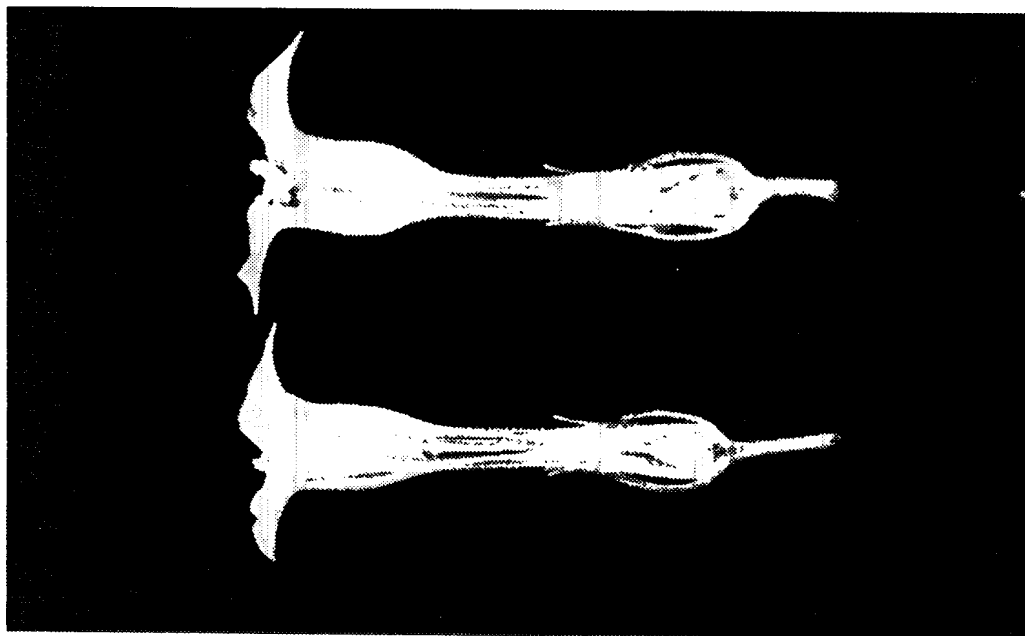


Fig. 11



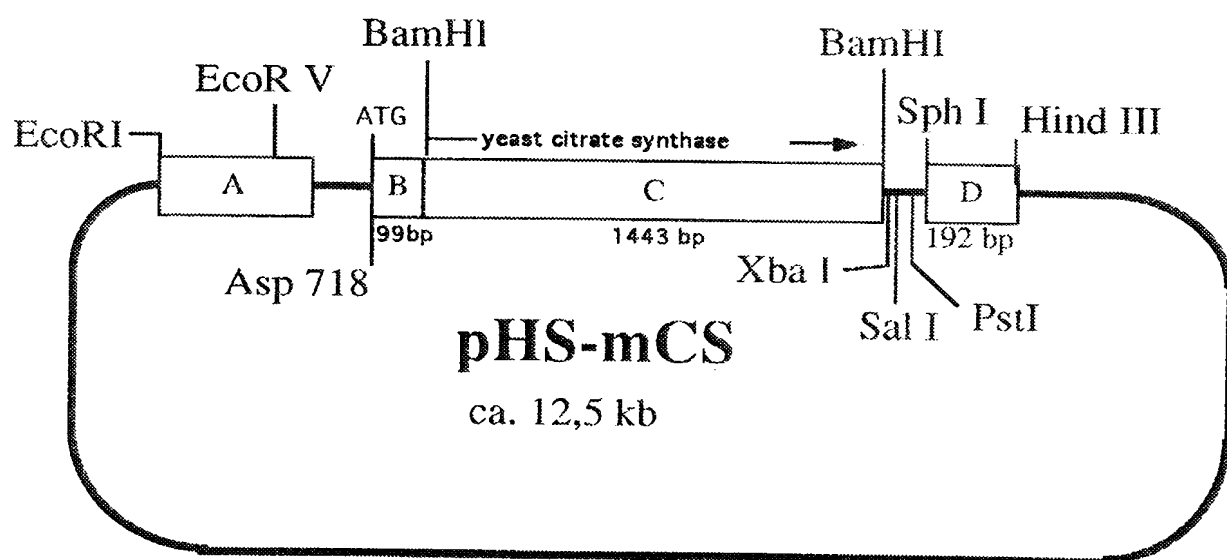


Fig. 12



Fig. 13



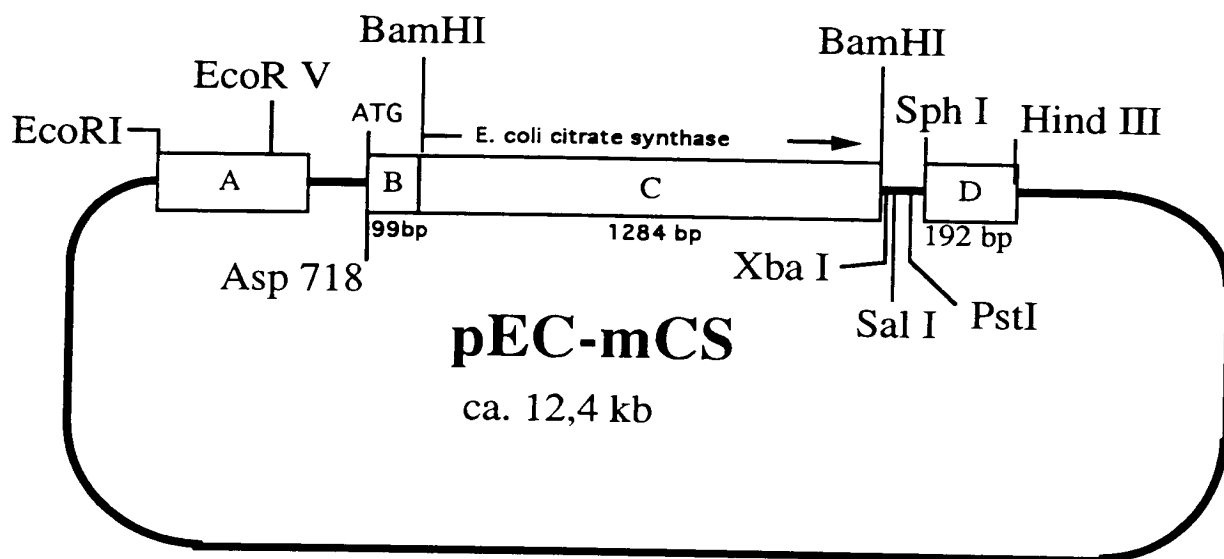
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**Fig. 14**



INTERNATIONAL SEARCH REPORT

International Application No.

/EP 95/00859

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/50 C12N15/82 C12N9/88 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|--|
| P,X | EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL 14 (4). 1995. 660-666., LANDSCHUETZE V., ET AL. 'Inhibition of flower formation by antisense repression of mitochondrial citrate synthase in transgenic potato plants leads to a specific disintegration of the ovary tissues' see the whole document --- | 1,2,5,8, 11, 14-18, 28,35, 39,40, 42-44, 46,47 |
| P,X | EMBL SEQUENCE DATABASE ACC. NO.X75082 RELEASE 39, 02-05-1994, LANDSCHUETZE V. ET AL., S.TUBEROSUM MRNA FOR MITOCHONDRIAL CITRATE-SYNTHASE see sequence --- -/-- | 1,2,5,8 |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

26 June 1995

Date of mailing of the international search report

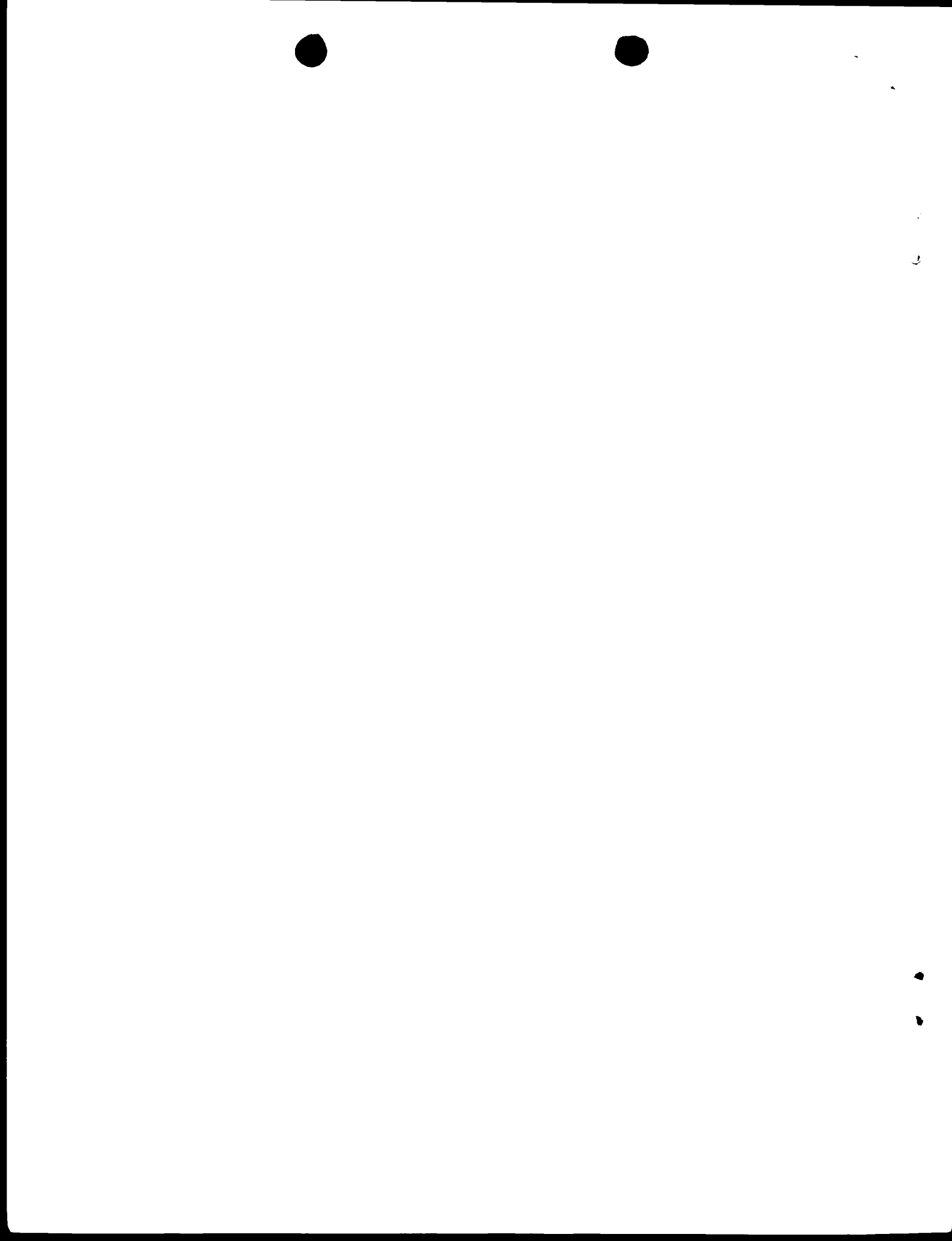
- 3. 07. 95

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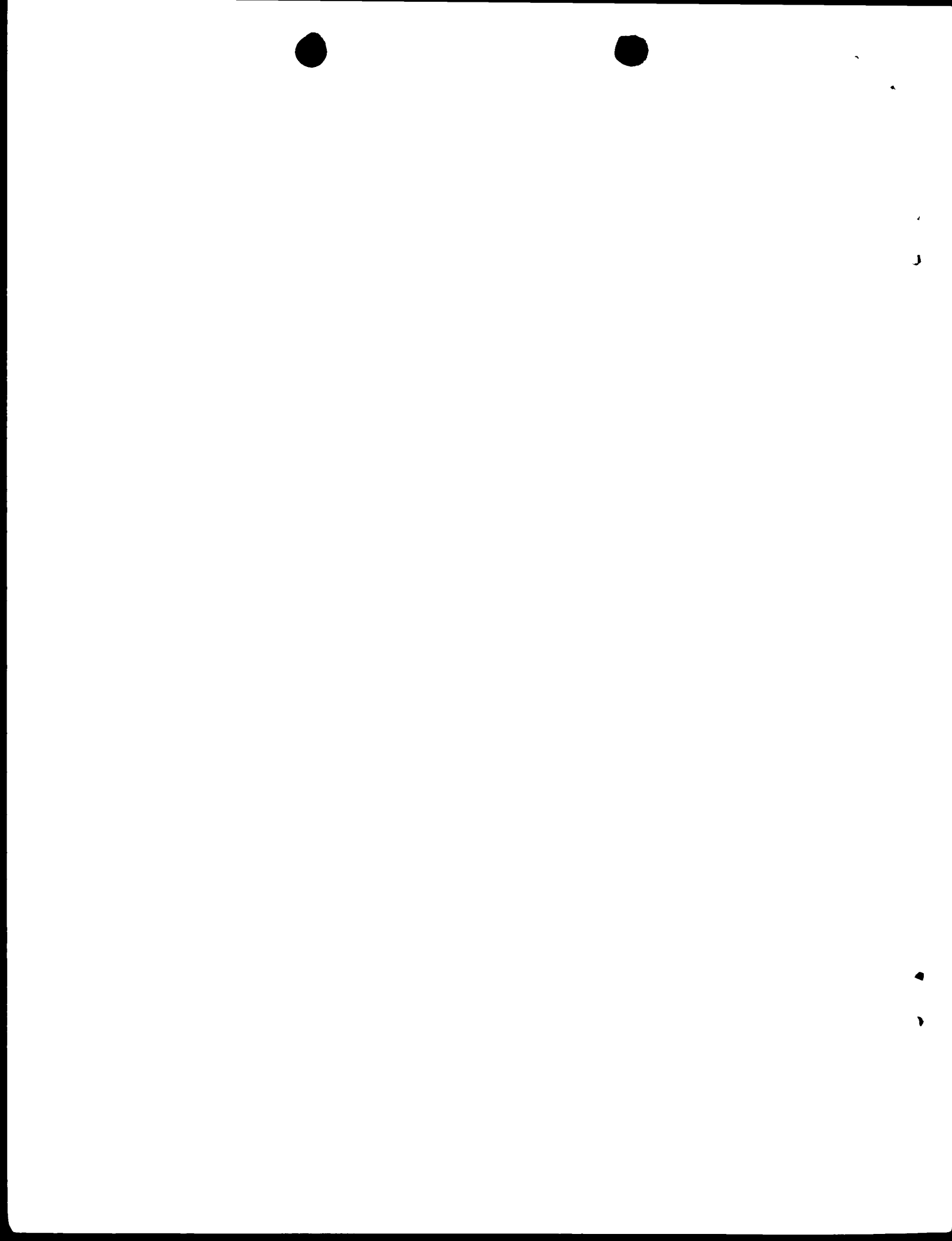
INTERNATIONAL SEARCH REPORT

International Application No.

/EP 95/00859

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|------------|--|-----------------------|
| A | WO-A-93 18154 (AGRONOMIQUE INST NAT RECH) 16 September 1993 see page 12, line 10 - line 24 --- | 19 |
| A | WO-A-93 12239 (ICI PLC) 24 June 1993 see page 9, line 13 - page 11, line 26 --- | 19 |
| A | PLANT MOL BIOL 13 (4). 1989. 411-418. , UNGER E A, ET AL. 'ISOLATION OF A COMPLEMENTARY DNA ENCODING MITOCHONDRIAL CITRATE SYNTHASE FROM ARABIDOPSIS-THALIANA.' see the whole document ----- | 1-10 |



INTERNATIONAL SEARCH REPORT

Invention on patent family members

International Application No

EP 95/00859

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO-A-9318154 | 16-09-93 | FR-A- 2688228 | 10-09-93 |
| | | EP-A- 0629242 | 21-12-94 |
| | | FI-A- 944042 | 12-10-94 |
| | | NO-A- 943266 | 01-11-94 |
| WO-A-9312239 | 24-06-93 | AU-B- 3165793 | 19-07-93 |



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | |
|--|-----------|---|
| (51) International Patent Classification $\bar{5}$: A01H 5/00, C12N 15/00 | A1 | (11) International Publication Number: WO 94/10831 (43) International Publication Date: 26 May 1994 (26.05.94) |
| (21) International Application Number: PCT/US93/10629 (22) International Filing Date: 5 November 1993 (05.11.93) (30) Priority data: 07/973,327 6 November 1992 (06.11.92) US (71) Applicant: WASHINGTON UNIVERSITY [US/US]; 724 South Euclid Avenue, St. Louis, MO 63110 (US). (72) Inventor: HO, Tuan-Hua, David ; 2033 Honey Ridge Court, Chesterfield, MO 63017 (US). (74) Agents: WEBER, Kenneth, A. et al.; Townsend and Townsend Kourie and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US). | | (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE) Published <i>With international search report.</i> |
| (54) Title: INDUCTION OF DWARFING AND EARLY FLOWERING USING GROUP 3 LEA PROTEINS (57) Abstract This invention relates to methods of inducing a dwarfed stature into plants by the recombinant introduction of a DNA sequence encoding a group 3 late embryogenesis abundant protein. In addition to dwarfism, the expression of this protein tends to confer early flowering, more branching, and stress resistance, in particular drought resistance, to the transformed plants. | | |

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| GA | Gabon | | | | |

INDUCTION OF DWARFING AND EARLY FLOWERING
USING GROUP 3 LEA PROTEINS

This invention was made with Government support under NSF Grant (or contract) No. DCB-9006591 and USDA Grant 91-03029. The Government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to methods of inducing a dwarfed stature into plants by the recombinant introduction of a DNA sequence encoding a group 3 late embryogenesis abundant protein. In addition to dwarfism, the expression of this protein tends to confer early flowering and stress resistance, in particular drought resistance, to the transformed plants.

The invention results in shorter, stronger and more branched plants and finds use in ornamental species such as petunia, geranium, and roses. In certain field crops such as wheat, barley and rice, shorter stems are beneficial to prevent lodging due to wind. Drought resistance has benefit in areas of low rainfall.

SUMMARY OF THE INVENTION

This invention provides a method of dwarfing a plant said method comprising: (a) transforming the plant with a gene encoding a group 3 late embryogenesis abundant (LEA) protein; and, (b) culturing the plant simultaneously with the expression of LEA protein in sufficient quantity to dwarf the plant wherein said dwarfing is measured by at least a 20% reduction in growth habit or average internodal length. It is preferred that the LEA protein is operatively linked to a constitutive promoter or an efficient promoter which permits continuous expression throughout the plant's life. Preferred sources of the LEA protein include but are not limited to the following plant species: *Hordeum vulgare*, *Triticum aestivum*,

Daucus carota, *Brassica napus*, *Gossypium hirsutum* and *glycine max*. The sequence of a preferred LEA is provided in Seq. ID No. 1. In addition to dwarfing, the gene will produce early flowering, more branching, a more pronounced or abundant vascular system and increased resistance to stress.

In addition to the above described methods, this invention provides for intact plants produced by the above method. The preferred species of plants include *Nicotiana tabacum*, *Petunia hybrida*, *Brassica napus*, *Arabidopsis thaliana* and *Oriza sativa*.

DEFINITIONS

The phrase "culturing the plant simultaneously with the expression of the LEA protein" refers to growing or cultivating the plant to maturity and using promoters which facilitate expression of the LEA protein during this growth process, the expression occurring generally throughout the plant in most if not all tissues.

The term "dwarfing" refers to a reduction in average size of a plant. It can be objectively measured in a variety of statistically significant ways. Typically by direct measurement of internodal length, overall height and diameter or ratio of different sizes of various plant organs.

The phrase "DNA sequence" refers to a single or double-stranded polymer of deoxyribonucleotide bases read from the 5' to the 3' end. It includes both genomic and cDNA, self-replicating plasmids, infectious polymers of DNA and non-functional DNA.

The term "group 3 LEA protein" includes the native and mutated forms of this family comprising structurally similar proteins each performing the same biological function in the native plant. It being further understood that polymorphism is likely and that minor allelic mutations both naturally occurring and deliberately induced are embraced by the term. Indeed, minor substitutions, deletions or additions to the primary amino acid sequence are possible and will have minimal impact upon the bioactivity of the enzyme. For

example, glutamine and asparagine, aspartate and glutamate residues are often interchangeable.

The phrase "intact plant" refers to complete plant having roots and above ground organs.

5 The phrase "mature plant" refers to a plant producing sexual organs, i.e., flowers.

 The phrase "operatively linked" refers to a functional covalent linkage between a promoter and a structural gene such that the host cell is able to transcribe
10 an RNA from the combination.

 The term "plant" includes whole plants, plant parts (e.g., leaves, stems, roots, seeds, etc.) and plant cells.

 The term "promoter" refers to a region of DNA upstream from the structural gene and involved in recognition
15 and binding RNA polymerase to initiate transcription.

 The term "transforming" refers to the introduction of a heterologous gene into a plant cell.

DETAILED DESCRIPTION

20 This invention provides for a method of dwarfing plants using a family of proteins termed Late Embryogenesis Abundant (LEA) proteins. A number of groups around the world have isolated cDNA and genomic clones encoding these proteins which are very abundant during late embryogenesis. They have
25 been generally described by Dure et al., 1989, *Plant Mol. Biol.* 12:475-486.

 Three groups of LEA proteins have been characterized. This invention utilizes group three of which the barley LEA, Hv A1 has been used herein. Other members of
30 group 3 are described in Table 1 of Dure et al., 1989, *supra*. They have been found in monocotyledonous plants such as barley and wheat as well as several dicotyledonous plants, such as rape seeds, carrot and cotton. They range in size from 14 to 66 kDa but are best characterized by their abundance in seeds
35 and unique tract of eleven repeating amino acids (see Fig. 4 of Dure ,et al. *Supra*).

 It has been shown recently that LEA protein genes can be induced by a plant hormone, abscisic acid (ABA), as

well as by several environmental stress conditions including drought, salinity, heat and cold (Hong et al., 1992, *Plant Mol. Biol.* 18:663-674). Surprisingly it has been found that this protein, when introduced into a plant by recombinant means and expressed in the mature plant will confer dwarfing as well as other beneficial properties to that plant. Such properties include early flowering and stress resistance.

A. General Methods

Generally, the nomenclature and general laboratory procedures with respect to recombinant DNA technology can be found in Sambrook, et al., *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 1989. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by references.

B. Sources of genes encoding group 3 LEA proteins

By using a number of standard procedures, one of skill can identify 3 LEA proteins in other species of plants. While the gene can be amplified directly from a mRNA extract using PCR, the first step is generally to produce a genomic or cDNA library.

In brief, genomic or cDNA libraries are prepared according to standard techniques as described, for instance, in Sambrook, *supra*. To construct genomic libraries, large segments of genomic DNA are generated by random fragmentation and are ligated with vector DNA to form concatemers that can be packaged into the appropriate vector. Two kinds of vectors are commonly used for this purpose, bacteriophage lambda vectors and cosmids.

In the present invention, cDNA libraries enriched for group 3 Lea gene sequences are generally used to screen for the desired genes. Preparation of appropriately enriched cDNA would involve the use of plant organs over expressing the LEA protein, e.g. aleurone layers and endosperm. Briefly,

mRNA from select tissue is isolated from total RNA and cDNA is prepared. Short chains of oligo d-T nucleotides are hybridized with the poly-A tails of the mRNA and serve as a primer for the enzyme, reverse transcriptase, which synthesizes a complementary DNA (cDNA) strand. The cDNA can be optionally enriched for the desired sequences using subtraction hybridization procedures by labelling the cDNA and hybridizing it with mRNA from tissue that does not express the desired mRNA according to the procedures. *Proc. Natl. Acad. Sci. U.S.A.* 81:2194-2198 (1984).

Unreacted cDNA is isolated and used to prepare a library for screening. To do this, a second DNA strand is synthesized using the first cDNA strand as a template. Linkers are added to the double-stranded cDNA for insertion into a plasmid or λ phage vector for propagation in *E. coli*.

Identification of clones harboring the desired sequences is performed by either nucleic acid hybridization or immunological detection of the encoded protein, if an expression vector is used. Typically, oligonucleotide probes specific for group 3 LEA protein genes are used.

Oligonucleotide probes useful for identification of other group 3 LEA genes can also be prepared from conserved regions of related genes in other species. By comparing the nucleotide sequences of the known group 3 LEA proteins, one simply identifies conserved sequences in the genes and uses those sequences as probes or a PCR primers to locate homologous sequences in genomic or cDNA libraries of other plants. Regions of nucleotide homology and amino acid identity are discussed in Dure et al. ,1989, *Plant Mol. Biol.* 12:475-486. For example, sequences 95 to 316, and 420 to 545 of Seq. ID. No. 1 are conserved and can be used to isolate other genes encoding type 3 LEA proteins.

The probes are typically used to identify sequences that hybridize under stringent conditions to ensure that the sequences are in fact related. Typically, stringent conditions suitable for finding related sequences would be performing the hybridization at room temperature using 1M salt.

More particularly, total RNA is isolated from aleurone layers and various organs using guanidine-HCl as described in Koehler and Ho, 1990, *Plant Cell* 2:769-783. Alternatively total RNA from developing endosperm can be prepared using the method of Rogers and Milliman, 1983 *J. Biol. Chem.* 258:8169-8174. For barley, poly A⁺ RNA is isolated from total RNA using embryo sources: embryos are collected at 30, 35, and 40 days post anthesis (DPA) and mature embryos from embryo-containing half-seeds hydrated and gently shaken in imbibition buffer plus 2 mM ABA (75 half-seeds/15 ml/14 cm petri plate) for 24 h at 20°C.

Genomic DNA can be isolated using a modified version of Sutton, 1974 *Biochem. Biophys. Acta* 336:1-10. Briefly, 25 g of frozen tissue is powdered in a coffee mill and gently homogenized in a mortar to a smooth slurry with 25 ml extraction buffer (0.2 M Tris-HCl, pH 8.5, 10 mM EDTA, 1% SDS). This mixture is extracted with phenol:chloroform and centrifuged (12,000 x g). The aqueous phase is adjusted to 0.5 M NaCl, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and centrifuged again. DNA is spooled through two volumes of ethanol. Spooled DNA is resuspended by overnight dialysis against TE (10 mM Tris HCl, pH 8.0, 1 mM EDTA), treated with 50 mg/ml RNase A (37°C, 1 h), adjusted to 150 mM NaCl, and extracted twice with chloroform. Genomic DNA was spooled from the final aqueous phase, dialyzed against TE, and stored at 4°C. Both DNA and RNA samples were quantitated by reading absorbance at 260 nm.

Poly(A)⁺RNA is used for cDNA synthesis (Krug and Berger, 1987, *Methods in Enzym.*, Vol. 152, pp. 316-325; Gubler, 1987, *Methods in Enzym.*, 152:325-335). Size fractionated (Huynh et al. 1985, pp. 49-78 of *DNA Cloning: A Practical Approach ILR Process*), EcoRI linkered cDNA (>1.5 kb) was ligated into the Lambda ZAP II phage vector (Stratagene) and packaged *in vitro* using Gigapack II Gold packaging extracts (Stratagene) following manufacturer's instructions. Approximately 10⁶ recombinant phage should be present in the resulting library. For screening, [³²P] labeled mRNA or PCR-generated probes are used. Hybridizations and washings were

performed essentially as described by Church and Gilbert (1984), *Genomic Sequencing* 8: 1991-1995. The largest positive clone is typically chosen for further analysis.

5 C. Cloning of the group 3 LEA Genes.

Once the gene or cDNA has been located, sufficient quantity of the gene or cDNA must be generated to facilitate subsequent recombinant manipulations. Although the sequences can be directly amplified by PCR, they are most commonly
10 replicated in an intermediate bacterial host. Most commonly in a bacteria of the genera: *Escherichia*, *Bacillus* or *Streptomyces*. Cloning for amplification of intermediate vectors is most preferred in *E. coli* because that organism is easy to culture and more fully understood than other species
15 of prokaryotes. The Sambrook manual contains methodology sufficient to conduct all subsequently described clonings in *E. coli*.

The cloning vectors contain an origin of replication suitable for directing replication in prokaryotes as well as
20 selectable markers. There are numerous examples of origin of replication markers in prokaryotes. *E. coli* replicons, which are the most closely studied, have origins of replication which are temperature dependent, high copy mutations, or those which constitutively sustain plasmid copies at only lower or
25 moderate levels. Examples of *E. Coli* origins of replication are ColE1 ori, R1 ori R, or pSC101 ori. Examples of selectable markers include for *E. Coli*: genes specifying resistance to antibiotics, i.e., ampicillin, tetracycline, kanamycin, erythromycin, or genes conferring other types of
30 selectable enzymatic activities such as beta-galactosidase, or the lactose operon; for *Bacillus subtilis*: the neomycin resistance gene from staphylococcal plasmid pUB110 and the chloramphenicol acetyltransferase gene from staphylococcal pC194; for *Streptomyces* sp.: the aminoglycoside
35 phosphotransferase (APH) gene encoding resistance to neomycin and kanamycin, the thiostrepton resistance gene, the hygromycin resistance gene, the viomycin resistance gene (see generally, Genetic Manipulation of *Streptomyces*, A Laboratory

Manual, Ed. David Hopwood, Cold Spring harbor Laboratories, Cold Spring Harbor, New York).

D. Expression vectors.

5 The desired expression vector consists of an expression cassette designed for plants and companion sequences upstream and downstream from the expression cassette. The companion sequences will be of plasmid or viral origin and provide necessary characteristics to the vector to
10 permit the vectors to move DNA from bacteria to the desired plant host.

 The basic bacterial/plant vector construct will preferably provide a broad host range prokaryote replication origin; a prokaryote selectable marker; and, for *Agrobacterium*
15 transformations, T DNA sequences for *Agrobacterium*-mediated transfer to plant chromosomes.

 Suitable prokaryote selectable markers include those identified in section C. Other DNA sequences encoding additional functions may also be present in the vector, as is
20 known in the art.

 The recombinant expression cassette will contain in addition to the LEA protein coding sequence, a promoter region, plant 5' untranslated sequences, initiation codon depending upon whether or not the structural gene comes
25 equipped with one, and a transcription and translation termination sequence. Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

 Promoters to direct m-RNA transcription should
30 operate effectively in plant hosts. Constitutive promoters are preferred. One such promoter is the nos promoter from native Ti plasmids, Herrera-Estrella et al., *Nature* 303:209-213, 1983. Others include the 35S and 19S promoters of cauliflower mosaic virus [CaMV] Odell et al., *Nature* 313:810-812, 1985, and the 2' promoter, Velten, et al., *EMBO J.* 3,
35 2723-2730, 1984 and the promoters from the ubiquitin, actin, opine synthetase, ribulose 1,5 bisphosphate carboxylase small subunit genes.

Transcription enhancers are elements upstream from the promoter site and include the "TATA box" as well as less characterized sequences which vary from species to species, Ha and An, *Proc Nat. Acad. Sci.* 85:8017-8021, 1988.

5 Polyadenylation tails, Alber and Kawasaki, 1982, *Mol. and Appl. Genet.* 1:419-434 are also commonly added to the vector construct to optimize high levels of transcription and proper transcription termination, respectively.

10 Polyadenylation sequences include but are not limited to the *Agrobacterium* octopine synthetase signal, Gielen et al., *EMBO J.* 3:835-846, 1984 or the nopaline synthase of the same species Depicker et al., *Mol. Appl. Genet.* 1:561-573, 1982.

15 Since the ultimate expression of the desired gene product will be in a eucaryotic cell (e.g., tobacco), it is desirable to determine whether any portion of the cloned gene contains sequences which will be processed out as introns by the host's splicosome machinery. If so, site-directed mutagenesis of the "intron" region may be conducted to prevent losing a portion of the genetic message as a false intron
20 code, Reed and Maniatis, *Cell* 41:95-105, 1985.

The LEA protein is preferably expressed at concentrations sufficient to induce the desired phenotypic changes. Typically the level of expression is about 0.5% of the total soluble protein but between .25 and 1% is
25 acceptable. The level of expression is measured by immunoblots (western blots) or by Northern hybridization assays where mRNA levels are assayed.

E. Transformation of plant cells

30 a. Direct Transformation

The LEA vector can be microinjected directly into plant cells by use of micropipettes to mechanically transfer the recombinant DNA. Crossway, *Mol. Gen. Genetics*, 202:179-185, 1985. The genetic material may also be transferred into
35 the plant cell by using polyethylene glycol, Krens, et al., *Nature*, 296, 72-74, 1982. Another method of introduction of nucleic acid segments is high velocity ballistic penetration by small particles with the nucleic acid either within the

matrix of small beads or particles, or on the surface, Klein, et al., *Nature*, 327, 70-73, 1987.

Yet another method of introduction would be fusion of protoplasts with other entities, either minicells, cells, lysosomes or other fusible lipid-surfaced bodies, Fraley, et al., *Proc. Natl. Acad. Sci. USA*, 79, 1859-1863, 1982.

The gene encoding the group 3 LEA protein may also be introduced into the plant cells by electroporation. (Fromm et al., *Proc. Natl. Acad. Sci. USA* 82:5824, 1985). In this technique, plant protoplasts are electroporated in the presence of plasmids containing the gene construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing the introduction of the plasmids. Electroporated plant protoplasts reform the cell wall, divide, and form plant callus.

b. Vectored Transformation

Cauliflower mosaic virus (CaMV) may be used as a vector for introducing the gene encoding the group 3 LEA protein into plant cells. (Hohn et al., 1982 "*Molecular Biology of Plant Tumors*," Academic Press, New York, pp.549-560; Howell, United States Patent No. 4,407,956). In accordance with the described method, the entire CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid is further modified by introduction of the group 3 LEA protein encoding genetic sequence into unique restriction sites of the viral portion of the plasmid. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants.

Another method of introducing the gene encoding group 3 LEA proteins into plant cells is to infect a plant cell with *Agrobacterium tumefaciens* previously transformed with the gene. Under appropriate conditions known in the art, the transformed plant cells are grown to form shoots or roots, and develop further into plants.

Agrobacterium is a representative genus of the gram-negative family Rhizobiaceae. Its species are responsible for

plant tumors such as crown gall and hairy root disease. In the dedifferentiated tissue characteristic of the tumors, amino acid derivatives known as opines are produced and catabolized. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes.

Heterologous genetic sequences can be introduced into appropriate plant cells, by means of the Ti plasmid of *Agrobacterium tumefaciens*. The Ti plasmid is transmitted to plant cells on infection by *Agrobacterium tumefaciens*, and is stably integrated into the plant genome. J. Schell, *Science* 237: 1176-1183, 1987.

Ti plasmids contain two regions essential for the production of transformed cells. One of these, named transferred DNA (T-DNA), is transferred to plant nuclei and induces tumor formation. The other, termed virulence region, is essential for the transfer of this T-DNA but is not itself transferred. The transferred DNA region, which transfers to the plant genome, can be increased in size by the insertion of the gene encoding group 3 LEA proteins without its ability to be transferred being affected. A modified Ti plasmid, in which the tumor-causing genes have been deleted, can be used as a vector for the transfer of the gene constructs of this invention into an appropriate plant cell.

Construction of recombinant Ti plasmids in general follows methods typically used with the more common bacterial vectors such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include but are not limited to "shuttle vectors", Ruvkun and Ausubel, 1981, *Nature* 298:85-88, promoters, Lawton et al., 1987, *Plant Mol. Biol.* 9:315-324 and structural genes for antibiotic resistance as a selection factor, Fraley et al., *Proc. Nat. Acad. Sci.* 80:4803-4807, 1983.

All plant cells which can be transformed by *Agrobacterium* and from which whole plants can be regenerated from the transformed cells can be transformed according to the invention to produce transformed intact plants which contain

and express the transferred gene encoding the group 3 LEA proteins.

There are two common ways to transform plant cells with *Agrobacterium*:

- 5 (1) co-cultivation of *Agrobacterium* with cultured isolated protoplasts, or
- (2) transformation of intact cells or tissues with *Agrobacterium*.

Method (1) requires an established culture system
10 that allows for the culturing protoplasts and subsequent plant regeneration from cultured protoplasts.

Method (2) requires (a) that the intact plant cells or tissues can be transformed by *Agrobacterium* and (b) that the transformed cells or tissues can be induced to regenerate
15 into whole plants. In both method (1) and (2), both a T-DNA and a *vir* region are required for transformation, but only the T-DNA genetic region is incorporated into the plant nuclear DNA.

After transformation of the plant cell or plant,
20 those plant cells or plants transformed by the Ti plasmid comprising the gene encoding the group 3 LEA protein can be selected by growing the plant cells on growth medium containing an appropriate antibiotic.

After selecting the transformed cells by virtue of
25 their ability to survive or to accumulate chlorophyll on the growth medium, one can confirm expression of the desired heterologous gene. Simple detection of RNA encoded by a cloned gene can be achieved by well known methods in the art, such as Northern blot hybridization, Maniatis.

30 After determination of the presence and expression of the desired gene products, whole plant regeneration is desired. All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention so that whole plants are
35 recovered which contain the transferred gene. Some suitable plants include, for example, species from the genera *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*,

Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hererocallis, Nemesia, Pelargonium,
5 *Panicum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Lolium, Zea, Triticum, Sorghum,*
and *Datura.*

It is known that practically all plants can be regenerated from cultured cells or tissues, including but not
10 limited to all major species of sugarcane, sugar beet, cotton, fruit and other trees, legumes and vegetables. Limited knowledge presently exists on whether all of these plants can be transformed by *Agrobacterium*. Species which are a natural plant host for *Agrobacterium* may be transformable *in vitro*.
15 Monocotyledonous plants, and in particular, cereals and grasses, are not natural hosts to *Agrobacterium*. Attempts to transform them using *Agrobacterium* have been unsuccessful until recently. Hooykas-Van Slogteren et al., *Nature* 311:763-764, 1984. There is growing evidence now that certain
20 monocots can be transformed by *Agrobacterium*. Using novel experimental approaches that have now become available, cereal and grass species may now be transformed.

Additional plant genera that may be transformed by *Agrobacterium* include *Ipomoea, Paasiflora, Cyclamen, Malus,*
25 *Prunus, Rosa, Rubus, Populus, Santalum, Allium, Lilium, Narcissus, Ananas, Arachis, Phaseolus* and *Pisum*.

Plant regeneration from cultured protoplasts is described in Evans et al., *Handbook of Plant Cell Cultures*, Vol. 1: (MacMillan Publishing Co. New York, 1983); and Vasil
30 I.R. (ed.), *Cell Culture and Somatic Cell Genetics of Plants*, Acad. Press, Orlando, Vol. I, 1984, and Vol. III, 1986.

Means for regeneration vary from species to species of plants, but generally a suspension of transformed protoplasts containing copies of the gene encoding the Group 3
35 LEA protein and other desired heterologous genes is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively, embryo formation can be induced from the protoplast suspension.

These embryos germinate as natural embryos to form plants. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is also advantageous to add glutamic acid and proline to the medium, especially for such species as corn and alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, on the genotype, and on the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

The mature plants, grown from the transformed plant cells, are selfed and non-segregating, homozygous transgenic plants are identified. The inbred plant produces seed containing the gene for the LEA protein product. These seeds can be grown to produce plants that exhibit the desired dwarfing characteristic.

The inbreds according to this invention can be used to develop hybrids or novel varieties embodying the desired traits. Such plants would be developed using traditional selection type breeding.

Once the plants expressing the group 3 LEA proteins are obtained, they are assayed for dwarfism, early flowering and stress resistance. Dwarfism is typically measured by a reduction in growth habit as compared to an appropriate control plant grown under similar conditions, often optimal conditions. Direct measurements of height or of internodal lengths are taken and statistical analysis performed. Typically this invention will provide a reduction often times a 30-50% reduction in either total height or internodal length.

Early flowering is also measured. Flowering is measured from germination or transplanting date to opening of the first flower. The control and group 3 LEA-transformed plants are grown under similar conditions, typically optimal and the number of days to anthesis is recorded and statistically compared. This invention will typically induce a 10-35% reduction in the days to flowering, more typically a 15 to 25% reduction in days to flowering.

An increase in branching is also conferred by these genes. Branching is measured by counting branch points appearing above the ground. One simply compares enough experimental and control plants so that a valid statistical comparison is possible. Typically branching is increased by at least 15% and preferably by 30% or more. In some studies the branching can be doubled.

Stress resistance is measured by comparison of time to leaf wilt between control and group 3 LEA-transformed plants grown under similar conditions, typically optimal. Differences are recorded and statistically compared. This invention will typically induce a 20% to 35% lengthening in time to cause wilting and/or a 20% to 50% reduction in leaf surface area. Finally a detectable strengthening of the vascular tissue has been noted.

All of the above parameters are objectively analyzed using standard statistical tools such as the T-test. Differences of $p \leq 0.05$ are considered real.

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

EXAMPLES

Transformation procedures:

Group 3 LEA protein from Barley described in Hong et al. 1988, *Plant Mol. Biol.* 11:495-506 was used to transform tobacco plants. The LEA protein was introduced using a *Agrobacterium* transformation vector, pMON 530, which is described by Rogers et al., 1987 *Methods in Enzymology* 153:253-278, and was provided by Monsanto Company. The features of this vector include: 1) the CAMV 35S promoter leads to constitutive expression of the foreign genes regardless the developmental stage of the transgenic plants, and 2) the vector also contains an antibiotic selection marker gene, NPTII (neomycin phosphotransferase), which confers resistance to kanamycin or its derivatives allowing an easy

selection for the transgenic plants. The full length HV Al cDNA was subcloned into pMON 530 at its EcoRI site. The transgenic tobacco (*Nicotiana tabacum* variety Xanthi) plants were obtained by the leaf disk transformation technique mediated by *Agrobacterium tumefaciens* (Horsch et al. *Science* 227:1229-1231, 1985).

The 35S promoter allowed the constitutive expression of HV Al in many tissues in the transgenic tobacco plants. HV Al RNA and protein were detected in the transgenic plants by Northern and Western analysis. Over a dozen individual transformants were obtained. Seven of them survived and produced progeny seeds. All of these seven plants were kanamycin resistant indicating the successful integration of the foreign genes in the transgenic plants. The R_0 transformants were allowed to self pollinate, and the R_1 seeds were obtained and designated below as a "family." R_1 progeny were segregated for the presence of HV Al. Western and dot blot analyses were employed to determine which of the R_1 progeny express HV Al protein. The R_1 progeny plants expressing the HV Al gene were more than 50% shorter in height than those without the HV Al gene. As a control, reduction in height was not observed in transgenic plants expressing TMV coat protein or movement protein under the same CAMV 35S promoter of the same vector. Therefore, the phenotypes observed are not the mere consequence of any plant transformation experiment.

a) The foreign genes were inserted into only one site of the tobacco genome:

At least five of the original transformants follow the single-locus dominant genetic segregation pattern (3:1 segregation ratio). The progeny seeds generated by selfing these plants were tested for kanamycin resistance during seed germination. The results of the segregation are provided in table 1.

Table 1.

| | Family of transgenic plants | # of Kan-resistant plants | # of Kan-sensitive plants | Ratio (KanR:KanS) |
|----|-----------------------------------|---------------------------------|---------------------------------|----------------------|
| 5 | I | 47 | 16 | 2.96:1 |
| | II | 146 | 46 | 3.17:1 |
| | IV | 256 | 67 | 3.80:1 |
| | V | 67 | 20 | 3.35:1 |
| 10 | X | 34 | 6 | 5.66:1 |
| | B | 98 | 26 | 3.77:1 |

b) Drastic morphological changes in transgenic plants, i.e. reduced height and more branching (bushy) compared to the wild-type controls:

The data from family II are presented in table 2. Similar data have been obtained with other families.

Table 2.

| | Plant group number | Height (cm) Ave + S.E. | # of branches/plant Ave |
|----|--------------------|---------------------------|----------------------------|
| 25 | #12 (control) | 208 ± 4 | 0.5 |
| | #16 (control) | 203 ± 4 | 1.1 |
| | #8 (transgenic) | 85 ± 3 | 3.8 |
| | #9 (transgenic) | 93 ± 3 | 2.3 |

c) Significantly earlier flowering (~15% sooner) in transgenic plants compared to the controls:

5 The data from family II are presented in Table 3.
Similar data have been obtained with other families.

Table 3.

| | | |
|----|-----------------|--|
| 10 | Plant group | number of days after seedling transplanting when flowers first appeared \pm S.E.) |
| | #12 (control) | 52 \pm 1 |
| 15 | #16 (control) | 52 \pm 1 |
| | #8 (transgenic) | 45 \pm 0 |
| | #9 (transgenic) | 46 \pm 1 |

20

d) Less wilting in leaves of transgenic plants as compared with the control:

25 Under standard greenhouse condition, more than half of
the lower leaves in control plants wilt after withholding
water for six days while the transgenic plants show no
apparent sign of wilting.

WHAT IS CLAIMED IS:

1. A method of dwarfing a plant said method comprising:
 - 5 (a) transforming the plant with a DNA sequence encoding a group 3 late embryogenesis abundant (LEA) protein; and
 - (b) culturing the plant simultaneously with the expression of the LEA protein at a level sufficient in
10 quantity to dwarf the plant wherein said dwarfing is measured by at least a 20% reduction in growth habit or average internodal length.
- 15 2. A method of claim 1 wherein the LEA protein gene is operatively linked to a constitutive promoter.
3. A method of claim 2 wherein the promoter is selected from the group consisting of: CaMV 35S, CaMV 19S, the ubiquitin gene promoter, the actin gene promoter, the opine
20 synthetase gene promoter and the ribulose 1,5 biphosphate carboxylase small subunit gene promoter.
4. A method of claim 1 wherein the LEA protein is selected from the LEA protein of a species of the group
25 consisting of: *Hordeum vulgare*, *Triticum aestivum*, *Daucus carota*, *Brassica napus*, *Gossypium hirsutum* and *glycine max*.
5. A method of claim 4 wherein the LEA protein has the amino acid sequence of Seq. ID. No. 1.
30
6. A method of claim 1 wherein the dwarfed plant is further characterized by early flowering.
7. A method of claim 1 wherein the dwarfed plant is
35 further characterized by drought resistance.

8. A method of claim 1 wherein the dwarfed plant is further characterized by at least 30% increase in branching at the base of the stem.

5 9. A method of claim 1 wherein the plant is selected from the group of plants comprising: *Nicotiana tabacum*, *Petunia hybrida*, *Brassica napus*, *Arabidopsis thaliana* and *Oriza sativa*.

10 10. An intact plant carrying a recombinant gene encoding a group 3 late embryogenesis abundant [LEA] protein, said protein being expressed at a concentration effective to induce a dwarfed stature in the mature plant wherein the dwarfed stature is measured by at least a 20% reduction in
15 growth habit or average internodal length.

11. A plant of claim 10 wherein the LEA protein is operatively linked to a constitutive promoter.

20 12. A plant of claim 11 wherein the promoter is selected from the group consisting of: CaMV 35S, CaMV 19S, the ubiquitin gene promoter, the actin gene promoter, the opine synthetase gene promoter and the ribulose 1,5 bisphosphate carboxylase small subunit gene promoter.

25 13. A plant of claim 10 wherein the LEA protein is selected from the LEA protein selected from the group consisting of: *Hordeum vulgare*, *Triticum aestivum*, *Daucus carota*, *Brassica napus*, *Gossypium hirsutum*, and *glycine max*.

30 14. A plant of claim 13 wherein the LEA protein has the amino acid sequence of Seq. ID. No. 1.

35 15. A plant of claim 10 wherein the dwarfed plant is further characterized by early flowering.

16. A plant of claim 10 wherein the dwarfed plant is further characterized by drought resistance.

17. A plant of claim 10 wherein the dwarfed plant is further characterized by at least 30% increase in branching at the base of the stem.

5 18. A plant of claim 10 wherein the plant is selected from the group of plants comprising: *Nicotiana tabacum*, *Petunia hybrida*, *Brassica napus*, *Arabidopsis thaliana* and *Oriza sativa*.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/10629

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A01H 5/00; C12N 15/00

US CL : 800/205; 435/172.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205; 435/172.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|---|-----------------------|
| Y | The EMBO Journal, Volume 7, No. 9, issued 1988, T. Schmulling et al, "Single Genes from Agrobacterium rhizogenes Influence Plant Development", pages 2621-2629, see page 2622. | 1-18 |
| Y | Plant Molecular Biology, Volume 11, issued 1988, B. Hong et al, "Cloning and Characterization of a cDNA Encoding a mRNA Rapidly Induced by ABA in Barley Aleurone Layers", pages 495-506, see page 502. | 1-18 |



Further documents are listed in the continuation of Box C.



See patent family annex.

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16 December 1993

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| (54) Title: PLANT CARRYING GENES CODING FOR ENZYMES OF THE PHYTOSTEROL BIOSYNTHESIS PATHWAY AND PROCESS FOR THE PRODUCTION OF SAME | | | |
| (54) Titre: PLANTE PORTANT DES GENES CODANT POUR DES ENZYMES DE LA VOIE DE BIOSYNTHESE DES PHYTOSTEROLS, ET PROCEDE D'OBTENTION | | | |
| (57) Abstract | | | |
| <p>Plant carrying in its genome one or more genes coding for one or more enzymes of the early stages of the phytosterol biosynthesis pathway. The genes are capable of coding for a mevalonate kinase, a farnesyl diphosphate synthetase or a mevalonyl 5-phosphate kinase and can be the genes ERG12, ERG20 and ERG8. The plants in which these genes are introduced in a stable manner have a number of advantages including increased development, enhanced productivity and regenerative ability.</p> | | | |
| (57) Abrégé | | | |
| <p>Plante portant dans son génomène un ou plusieurs gènes codant pour une ou plusieurs enzymes des étapes précoces de la voie de biosynthèse des phytostérols. Ces gènes peuvent coder pour une mévalonate kinase, une farnésyl diphosphate synthétase ou une mévalonyl 5-phosphate kinase et peuvent être les gènes ERG12, ERG20 et ERG8. Les plantes dans lesquelles ces gènes sont insérés de manière stable présentent de nombreux avantages tels qu'un développement plus important et une amélioration de leur productivité et de leur capacité à régénérer.</p> | | | |

UNIQUEMENT A TITRE D'INFORMATION

Codes utilisés pour identifier les Etats parties au PCT, sur les pages de couverture des brochures publiant des demandes internationales en vertu du PCT.

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Plante portant des gènes codant pour des enzymes de la voie de biosynthèse des phytostérols , et procédé d'obtention.

La présente invention a pour objet des plantes
5 portant des gènes codant pour une ou plusieurs des enzymes des étapes précoces de la voie de biosynthèse des phytostérols .

Elle est également relative à un procédé d'obtention desdites plantes .

10 L'acide mévalonique (AMV) est un précurseur des unités isoprènes en C₅ qui sont elles-mêmes à la base de la biosynthèse d'une part des phytostérols , et d'autre part de nombreuses molécules présentant une activité physiologique fondamentale dans le
15 métabolisme, la prolifération et la différenciation cellulaires des végétaux : régulateurs de croissance, caroténoïdes , chaîne phytol de la chlorophylle, ubiquinone.

La première étape de métabolisation de l'AMV est sa phosphorylation par la mévalonate kinase (MK). L'AMV
20 est ensuite converti en isopentényl pyrophosphate (IPP), molécule dont dérivent les cytokinines (CKs), et qui constitue l'unité isoprénique d'enchaînement menant en particulier à la synthèse des gibbéréllines (GAs) et
25 de l'acide abscissique (ABA). Les voies de biosynthèse simplifiées de ces trois catégories de phytohormones sont schématisées sur les figures 1A et 1B . Ces phytohormones sont particulièrement intéressantes du fait de leur implication dans les phénomènes de
30 régénération;

Le point de départ de la voie de biosynthèse des GAs est l'AMV. Une suite de réactions aboutit ensuite à la cyclisation du géranyl-géranyl pyrophosphate (GGPP) et à la formation de kaurène qui est oxydé et réarrangé

pour donner l'aldéhyde de GA_{12} (ROBERTS et HOOLEY, Plant Growth Regulators 1988 ed: Blakie, Chapman & Hall, New York,) , produit dont dérivent les diverses GAS.

5 Les sites de synthèse GAS se situent chez les très jeunes feuilles, dans les bourgeons en activité, aux extrémités des racines et au niveau des embryons . Leur circulation se fait par la voie libérienne ; les GAS sont souvent liées à des sucres et sont libérées au
10 niveau des sites d'action.

Les principales propriétés des GAS sont :

- un effet net sur l'élongation des entrenoeuds. Cet effet peut aussi se produire sur les pédoncules floraux, ce qui permet d'obtenir soit une
15 plus grande précocité soit des inflorescences plus développées ,

- un effet stimulant sur le métabolisme, car elles favorisent la synthèse d'enzymes hydrolysantes . Les GAS agissent également sur la teneur en auxines qui
20 est souvent augmentée soit par stimulation des synthèses soit par inhibition des auxines-oxydases,

- une action complexe sur les processus impliqués dans la floraison; elles seraient plutôt inhibitrices de l'induction florale en particulier chez
25 les arbres fruitiers. Par contre, pour des espèces ayant besoin de froid pour fleurir, une application de GA_3 pourrait permettre la floraison en absence de froid,

- une action également complexe sur les
30 phénomènes de levée de dormance ,

- enfin , dans le domaine de l'organogenèse, les GAS se comportent en antagonistes .

Les cytokinines sont des adénines substituées dérivant des purines.

Les ARN de transfert constituent une source potentielle de CKs chez les végétaux, mais cette voie ne serait cependant pas la seule puisqu'il est admis aujourd'hui, que la voie privilégiée de biosynthèse de CKs provient du métabolisme de l'AMV. Dans les deux voies proposées, l'IPP joue un rôle stratégique. Dans ce cas, il se condense avec une adénosine-5'-monophosphate, alors que dans l'autre cas, il se condense avec un résidu adénine issu des ARN de transfert.

Les CKs sont très actives et présentent de nombreuses actions dont les principales sont:

- un effet très net sur la division cellulaire;
- un rôle très net également dans l'organogenèse où elles sont antagonistes de la rhizogenèse ,
- une action stimulante sur le métabolisme, d'une part en favorisant les synthèses protéiques et d'autre part, en protégeant les métabolites contre les actions des enzymes hydrolysantes. Cet effet provoque un retard de sénescence au point que des feuilles traitées avec des CKs peuvent entrer en concurrence avec des feuilles plus jeunes au niveau des corrélations morphogènes de la plante,
- un effet antagoniste de la dominance apicale; des bourgeons axillaires traités par des CKs entrent en croissance et en compétition avec l'axe terminal.

L'ABA est synthétisé à partir de l'AMV via le farnésyl pyrophosphate (FPP) (MOORE T.C. Biochemistry and physiology of plant hormones ed: Springer Verlag, New York Heidelberg, Berlin, 1979) . Une autre voie menant à la synthèse de cet inhibiteur a également été décrite. Elle dérive des caroténoïdes et en particulier, de la violaxanthine.

Les sites de synthèse de l'ABA dans la plante

sont multiples . En effet , lorsque des feuilles isolées, des tiges ou des racines sont placées en condition de "stress" hydrique, une augmentation du taux d'ABA est observée .

5 L'ABA est également présent dans les fruits et dans les graines .

L'ABA joue un rôle majeur dans la régulation de la fermeture des stomates.

10 L'ABA joue également un rôle dans les phénomènes de dormance observés au niveau de bourgeons ou de graines. Il exerce aussi une action complexe sur la floraison. Il semble que l'ABA soit très impliqué dans le contrôle de l'abscission des fruits.

15 La position clé occupée par le FPP dans les voies de biosynthèse des GAS et de l'ABA d'une part et, d'autre part, la nature isoprénique des groupements substituants des CKs naturelles montrent toute l'importance de la MK dans la biosynthèse de ces différents types de régulateurs de croissance.

20 Ces voies sont résumées sur les diagrammes des figures 1A et 1B .

Des plantes dans lesquelles ont été intégrés des gènes codant pour des enzymes intervenant dans la voie de biosynthèse des phytostérols ont déjà été décrites.

25 La demande Internationale WO-9 113 078 (AMOCO) décrit des plantes transgéniques portant dans leur génome des fragments d'ADN codant pour la géranylgéranyle pyrophosphate synthase (GGPP).

30 Cette étape , dans la voie de biosynthèse des caroténoïdes est située en aval par rapport aux étapes précoces de la voie de biosynthèse des stérols.

Un compte-rendu de l'"Annual Meeting of the American Society of Plant Physiologists" (Albuquerque, Nouveau Mexique, 28 Juillet - 1er Août 1991) , concerne

des plantes dans lesquelles a été intégré un gène de hamster codant pour la 3-hydroxy-3-méthylglutaryle co-enzyme A réductase (HMGR).

5 On notera que cette étape est en amont de l'étape de transformation de l'acide mévalonique, dans laquelle la HMGR n'intervient pas.

Des gènes de nature différente ont aussi déjà été insérés dans diverses plantes au moyen principalement de dérivés de plasmides Ti . Cette
10 technique qui a notamment été décrite par KLEE et al.(Ann. Rev. , Plant Physiol 38, 1987, 467-486) , permet l'introduction d'un nombre limité de gènes en une seule opération.

Néanmoins, cette méthode très récente reste
15 inapplicable pour différentes espèces ou variétés, en particulier les graminées .

En outre , même si l'insertion du gène est rendue possible , il est nécessaire qu'il soit exprimé afin de produire ses effets dans les cellules
20 végétales. Ces techniques bien qu'évoluant rapidement, ne permettent pas d'introduire n'importe quel gène dans n'importe quelle cellule et de nombreux échecs d'expression de gènes étrangers à la plante ont été enregistrés par diverses équipes .

25 Outre les problèmes d'insertion des gènes et la fabrication d'un vecteur adéquat , on peut être confronté à des problèmes d'expression des gènes transférés dans les cellules végétales aboutissant à une absence d'expression ou une très faible expression
30 et à une activité inexistante du produit du gène recherché. Ces problèmes d'expression peuvent être dus notamment à l'incapacité du promoteur , qui est un des facteurs intervenant dans la régulation de l'expression du gène , à induire la synthèse d'ARN messenger.

Il peut aussi s'agir d'une incapacité de la plante à modifier de manière adéquate le produit de traduction de l'ARN messager .

5 Un autre problème réside dans les difficultés rencontrées dans la régénération de certaines espèces ou variétés ou même dans l'impossibilité à effectuer une telle opération . Certaines espèces de légumineuses et de graminées sont récalcitrantes à la régénération par des méthodes classiques .

10 Ainsi , l'analyse de l'état de la technique met en évidence que , malgré les résultats intéressants obtenus ces dernières années dans le domaine de la biologie moléculaire végétale , de nombreux obstacles s'opposent au transfert de gènes et à l'expression de
15 ces gènes dans des espèces ou des variétés spécifiques de plantes présentant des intérêts agronomiques

La demanderesse s'est donc attachée à trouver des moyens permettant d'insérer dans des plantes d'intérêt agronomique des gènes codant pour les enzymes
20 des étapes précoces des voies de la biosynthèse des phytostéroïdes et permettant l'expression desdits gènes dans les plantes.

La demanderesse s'est d'autre part attachée à mettre au point un procédé permettant de modifier
25 l'équilibre hormonal endogène des plantes afin d'améliorer la capacité de régénération des espèces difficiles à régénérer .

La demanderesse a ainsi montré d'une manière surprenante qu'il est possible d'insérer de manière
30 stable dans le génome de ces plantes , des gènes codant pour les voies de biosynthèse des phytostéroïdes , et que les plantes ainsi obtenues présentent de nombreux avantages, tels qu'un développement plus important, une productivité améliorée et une amélioration de leur

capacité à régénérer .

La présente invention a donc pour objet une plante caractérisée en ce qu'elle porte dans son génome un ou plusieurs gènes codant pour une ou plusieurs enzymes des étapes précoces de la voie de biosynthèse des phytostérols , lesdits gènes soit ne se retrouvant pas dans la plante native, soit étant originaires de ladite plante , auquel cas ils sont présents en un nombre de copies supérieur à celui de la plante native ou ils sont modifiés dans leur structure ou leur fonction par rapport à ceux de la plante native .

On entend, dans la présente demande , par étapes précoces de la voie de biosynthèse des phytostérols , la chaîne de réaction biochimique par laquelle l'acide mévalonique est, comme indiqué sur les figures 1A et 1B, transformé en farnésyl diphosphate.

Avantageusement , l'étape pour laquelle codent ce ou ces gènes est la transformation de l'acide mévalonique en mévalonyl 5-phosphate . Dans ce cas, la plante peut porter et/ou exprimer un ou plusieurs gènes codant pour une ou plusieurs mévalonate kinases (MK) , et en particulier le gène ERG12 , ou l'un de ses dérivés ou fragments exprimant une activité mévalonate kinase . Ce gène a été isolé chez la levure par Oulmouden et Karst (Gene , 88, 253-257, 1990) et séquencé par les mêmes auteurs (Curr Genet , 19, 9-14, 1991).

Il peut notamment s'agir de gènes codant pour l'activité mévalonate kinase chez les crucifères et en particulier chez Arabidopsis ou chez le colza.

Lesdites plantes peuvent porter aussi un ou plusieurs gènes codant pour une farnésyl diphosphate synthétase, tels que le gène ERG 20 de levure (Chambon et al., Curr. Genet, 18, 41, 1990) ou l'un de ses

dérivés ou fragments exprimant une activité farnésyl diphosphate synthétase.

Elles peuvent également porter un ou plusieurs gènes codant pour une AMVP kinase (mévalonyl 5-P-kinase), tels que le gène ERG 8 (Tsay et al., Mol. Cell. Biol., 11, n°2, 620, 1991) ou l'un de ses dérivés ou fragments exprimant une activité AMVP-kinase.

Les étapes des voies de biosynthèse dans lesquelles interviennent la farnésyl diphosphate synthétase et l'AMVP-kinase sont respectivement , comme indiqué sur la figure 1B, les étapes de transformation du diméthylallyl pyrophosphate (DMAPP) en farnésyl pyrophosphate (FPP) et de transformation du mévalonyl 5-phosphate (AMVP) en mévalonyl 5-pyrophosphate (AMVPP).

Le ou les gènes peuvent avoir été obtenus par clonage à partir d'ADN extrait d'organismes ou de virus vivants ou de toute autre matière biologique , ou peuvent avoir été synthétisés.

Lesdites plantes, portant ces gènes codant pour un ou plusieurs enzymes des étapes précoces de la voie de biosynthèse des phytostérols , sont avantageusement des plantes appartenant à la catégorie des plantes oléoprotéagineuses , et en particulier de la famille des crucifères telle que l'espèce colza ou de la famille des Fabacées telle que l'espèce soja .

Ladite plante peut aussi être une plante des espèces tournesol , aubergine (*Solanum melongena* var.) ou *Vigna radiata*.

De manière avantageuse, le ou les gènes sont placés sous le contrôle d'un promoteur inséré à proximité du ou des gènes, de manière à permettre l'expression de leurs produits biologiquement actifs .

La présente invention a d'autre part pour objet

un vecteur pour l'insertion d'au moins un gène précité dans des cellules végétales, caractérisé en ce qu'il comprend :

- la séquence du gène à insérer ,
- 5 - une ou plusieurs séquences du type promoteur en amont du gène à insérer ,
- une ou plusieurs séquences du type terminateur en aval du gène à insérer ,
- un ou plusieurs marqueurs permettant la
- 10 sélection des cellules végétales dans lequel le gène s'est inséré, et
- des séquences d'un plasmide Ti permettant le transfert desdits gènes et marqueurs d'une bactérie vers une cellule végétale , lesdits gènes et marqueurs
- 15 étant orientés et insérés de manière à permettre leur expression.

Préférentiellement , le gène à insérer code pour une ou plusieurs enzymes des étapes précoces de la voie de biosynthèse des phytostérols . Ce peut-être un gène

20 codant pour la mévalonate kinase et en particulier le gène ERG12, précédemment cité .

Les marqueurs permettant la sélection des cellules dans lesquelles le ou les gènes se sont insérés peuvent être toute activité enzymatique

25 permettant la sélection directe ou indirecte des cellules dans lesquelles il y a eu insertion, et en particulier des marqueurs de résistance à des antibiotiques tels que le marqueur de résistance à la kanamycine chez les végétaux , ou des marqueurs dont

30 l'activité enzymatique est utilisée pour colorer les cellules végétales tels que le gène lacZ.

Un tel vecteur d'insertion est notamment , le plasmide pFAB2 portant le gène ERG12 déposé sous le n° I-1176 du 14 Février 1992 auprès de la Collection

Nationale de Culture des Microorganismes.

Un vecteur peut être aussi un dérivé de ce plasmide et dans lequel le gène ERG12 a été remplacé par un autre gène codant pour une ou plusieurs des enzymes de la voie de biosynthèse des stérols .

Un autre objet de la présente invention est un procédé de traitement des plantes destiné à améliorer leur productivité , et en particulier leur performance de croissance ou leur précocité de floraison , par insertion dans leur génome d'un ou plusieurs gènes codant pour une ou plusieurs enzymes des étapes précoces de la voie de synthèse des phytostérols .

L'insertion du ou des gènes est préférentiellement effectuée par transfert dans des cellules individualisées ou dans des groupes de cellules d'un vecteur tel que décrit précédemment , éventuellement en présence d'un autre vecteur de type plasmide Ti ayant des fonctions permettant ce transfert.

Préférentiellement , de tels vecteurs seront dans des souches d'Agrobactérium .

Le transfert peut aussi être effectué , en absence de vecteurs ayant de telles fonctions , par une technique physique appropriée telle que l'électroporation.

Le transfert du vecteur à partir d'Agrobactérium tumefasciens dans des cellules végétales est effectué par toute méthode connue en soi par l'homme du métier et avantageusement par inoculation de disques foliaires.

Les cellules ainsi inoculées sont alors sélectionnées pour la présence des vecteurs et les plantes sont régénérées par les méthodes connues en soi et notamment décrites dans "Plant molecular physiology

manual " (Gelvin et Schilperdort, Kluwers Academic Publishers , 1988). Selon la présente invention, on a obtenu de bons résultats en transférant le gène ERG12 de levure par le système Agrobacterium.

5 La présente invention est illustrée par les exemples qui suivent avec référence aux dessins annexés , dans lesquels:

- Les figures 1A et 1B , déjà citées , sont des diagrammes illustratifs de données connues ;

10 - la figure 2 est un diagramme résumant le procédé d'obtention du plasmide pFAB2 portant le gène ERG12.

15 - la figure 3 est une carte de restriction partielle du fragment BamHI- BamH-I de 2,8 kilobases (kb) contenant le gène ERG12.

- les figures 4 et 5 sont des cartes de restriction respectivement des plasmides et pLBR19 et pFL44 .

20 - la figure 6 résume l'obtention du plasmide pFAB1 à partir du plasmide pLBR19 par insertion du fragment BamH I-BamHI contenant le gène ERG12 .

25 - la figure 7 est la photographie d'un gel d'électrophorèse des produits d'hydrolyse de pAO11 par BamH-1 (piste 1), de pFAB1 par BamH1 (piste 2), de pLBR19 par Sma1 (piste 3), de pLBR19 par Pst1 (piste 4) , de pAO11 par Pst1 et Sma1 (piste 5) et de l'ADN témoin du bactériophage lambda hydrolysé par Hind-III. Les deux flèches à gauche de la photographie, correspondent respectivement aux positions des bandes
30 de 1,8 kb et 0,8 kb .

- la figure 8 représente le carte du plasmide pBin19 .

- la figure 9 représente l'obtention du plasmide pFAB2 à partir des plasmides pBin19 et pFAB1.

- la figure 10 est une photographie d'un gel d'électrophorèse coloré par du bromure d'éthidium et révélé sous rayons ultraviolets d'ADN du bactériophage lambda hydrolysé par Hind III (piste 1) , et des produits d'hydrolyse de pFAB1 par BamH1 (piste 2), de pFAB2 par BamH1 (piste 3) , de pBin19 par Sall (piste 4), de pBin19 par SacI (piste 5), et de pFAB1 par XhoI et SacI (piste 6). Les flèches à droite de la photographie indiquent les positions respectives des bandes de taille 1,8 kb et 0,8 kb.

- les figures 11 et 12 correspondent à des diagrammes en chromatographie sur couche mince de gel de silice, de surnageants de culture de la souche d'*Escherichia coli* 71/18 portant respectivement les plasmides pFL44 (figure 11A), pAO11 (figure 11B) et pFAB1 (figure 11C) et de la souche d'*Agrobacterium tumefaciens* LBA4404 portant respectivement les plasmides pBin19 (figure 12A) et pFAB2 (figure 12B) . Les abréviations AMV et AMVP sur les figures , correspondent respectivement à la position des pics de l'acide mévalonique et au mévalonyl 5 P.

- la figure 13 correspond à un autoradiogramme d'un Southern blot dont l'hybridation a été effectuée par une sonde portant le gène ERG12. Les pistes 1, 2 et 3 correspondent respectivement aux plasmides pAO11 (témoin positif), à l'ADN total extrait d'une plante " témoin H₂O" , et à l'ADN total extrait d'une plante transformée .

- la figure 14 est la séquence partielle du gène ERG12, les localisations des oligonucléotides ON₁ et ON₂ et du site de restriction BamHI étant indiquées sur cette figure .

- la figure 15 est un gel d'électrophorèse de produits de réaction d'amplification par la technique

PCR . Les pistes 1 à 6 correspondent à l'amplification à partir d'ADN total extrait de tabacs transformés , la piste 7 correspond à l'amplification à partir de l'ADN génomique de levure , la piste 8 correspond à l'amplification avec l'amorce ON1 seule , la piste 9 correspond à l'amplification avec l'amorce ON2 seule , la piste 10 correspond à l'amplification à partir de l'ADN total extrait d'un tabac " témoin H₂O" , la piste 11 correspond à un témoin de non-contamination (eau stérile) et la piste 12 correspond au plasmide pBlueScript hydrolysé par HpaII (marqueur de poids moléculaire). La taille des bandes d'hydrolyse du marqueur est indiquée à droite de la photographie .

- La figure 16 est une photographie d'un gel d'agarose après amplification par la technique PCR et hydrolyse par BamHI respectivement d'ADN total extrait d'une plante transformée (piste 1) et d'ADN génomique de levure (piste 2). La piste non marquée à gauche de la piste 1 est de l'ADN du plasmide pBluescript hydrolysé par HpaII .

EXEMPLES

Matériel et méthodes utilisés dans les exemples .

I. Techniques de biologie moléculaire.

1. Matériel biologique

1.1- Souches bactériennes.

Les souches bactériennes utilisées soit comme source de vecteur, soit comme souche hôte des plasmides recombinants, dérivent d'*Escherichia coli* K12. La souche Hb101 (BOYER et ROULLAND-DUSSOIX - J. Mol. Biol. 41, 1969, 469-472), résistante à la streptomycine a été fournie par le Laboratoire de biologie de la rhizosphère de l'Institut National de la Recherche Agronomique (INRA) de Versailles et la souche 71/18 (MESSING et al. Proc. Natl. Acad. Sci. USA, 74,

1977,3642-3646) par le Laboratoire de Biochimie et Génétique des Microorganismes de l'Université de Poitiers.

5 La souche HB101 utilisée pour la mobilisation du vecteur de transfert vers *A. tumefaciens* contient le plasmide pRK2013 qui apporte les résistances à la kanamycine et à la streptomycine (BEVAN, Nucleic Acids Research ,12, 8711-8721 1984).

10 La souche d'*A. tumefaciens* provient également du Laboratoire de Biologie de la Rhizosphère de l'Institut National de la Recherche Agronomique (INRA) de Versailles . Il s'agit de la souche LBA4404 (HOEKMA et al., Nature , 303,179-180 1983), résistante à la streptomycine et à la rifampicine. Elle dérive de la
15 souche Ach5 et possède un plasmide Ti délété de la région T. Elle contient en plus le vecteur binaire de transfert Bin 19 (BEVAN, 1984, précédemment cité) .

Les souches d'*E. coli* sont cultivées à 37°C et *A. tumefaciens* est cultivé à 28°C.

20 1.2. Les plasmides .

Trois plasmides ont été utilisés pour la construction du vecteur de transfert . Ils sont décrits ci-dessous :

25 - Plasmide pAO11 : obtenu par clonage d'un fragment BamH I-BamH I de 2,8 kb contenant le gène ERG12 de levure, dans le plasmide pFL44

30 - Plasmide pLBR19 : il présente l'avantage de posséder de nombreux sites uniques de restriction insérés entre les séquences promoteur et terminateur de l'ARN 35S du virus de la mosaïque du chou-fleur . Ce plasmide comporte également un gène de résistance à l'ampicilline . Il a été fourni par le Laboratoire de Biologie de la rhizosphère de l'INRA de Versailles.

- Plasmide pBin19 : ce vecteur autonome, capable

de se répliquer dans *E.coli* et dans *A.tumefasciens*,
dérive du plasmide pRK252 modifié par insertion d'un
gène de résistance à la kanamycine provenant de
Streptococcus faecalis (BEVAN, 1984, précédemment
5 cité). Il comporte les séquences bordures droite et
gauche de l'ADN-T du plasmide pTi37.

Ont été insérés entre ces deux séquences :

(1) un fragment d'ADN de 440 pb provenant du
phage M13 et qui contient la région de complémentation
10 de la β -galactosidase (Lac Z) dans laquelle a été
insérée une séquence qui porte plusieurs sites de
clonage.

(2) un gène chimérique conférant une résistance
à la kanamycine chez les végétaux, construit par
15 insertion de la région codante d'une aminoglycoside
transférase de type II (provenant de Tn5) entre les
séquences régulatrices du gène de la nopaline
synthétase.

Toute séquence d'ADN insérée au niveau du site
20 de clonage multiple, compris dans la région délimitée
par les séquences bordures droite et gauche de l'ADN-T,
peut donc être transférée et intégrée dans le génome
d'une plante, le transfert étant induit , à distance ,
par les fonctions de virulence portées par un plasmide
25 Ti hébergé par la même souche d'*A.tumefasciens* . Le
système de sélection est assuré par la co-intégration
du gène de résistance à la kanamycine.

Les plasmides pAO11 et pFL44 ont été fournis par
le Laboratoire de Biochimie et de Génétique des
30 Microorganismes de l'Université de Poitiers , tandis
que le plasmide pLBR19 a été fourni par le Laboratoire
de Biologie de la Rhizosphère de l'Institut National de
la Recherche Agronomique (INRA) de Versailles .

2. Milieux de culture.

- Milieu L. Broth , utilisé pour la culture des souches d'E.coli :

- | | | |
|---|------------------------------|------|
| | - Tryptone (Biokar) | 10 g |
| 5 | - Extrait de levure (Biokar) | 5 g |
| | - NaCl | 5 g |
| | - Eau distillée | 1 l |

Le pH du milieu est amené à 7 avant stérilisation (120°C pendant 20 min) . Ce milieu est utilisé liquide ou solidifié par de l'agar à 20 g/l . La gélose molle L.Broth utilisée dans les expériences de transformation est obtenue par addition d'agar à la concentration de 8 g/l .

- Milieu AP, utilisé pour la culture des agrobactéries :

- | | | |
|----|-----------------------------------|---------|
| | - Extrait de levure (Biokar) | 5 g |
| | - Hydrolysate de caséine (Sigma) | 0,5 g |
| | - Mannitol | 8 g |
| | - $(\text{NH}_4)_2\text{SO}_4$ | 2 g |
| 20 | - NaCl | 5 g |
| | - Eau distillée | 1 litre |

Le pH du milieu est amené à 6,6 avant stérilisation (115°C pendant 20 min). Ce milieu est utilisé liquide ou solidifié par de l'agar à la concentration de 15 g/l .

Les antibiotiques sont utilisés aux concentrations suivantes (mg.l^{-1}):

| | E. coli | A.tumefasciens |
|---------------|---------|----------------|
| ampicilline | 100 | - |
| kanamycine | 50 | 50 |
| 5 rifampicine | - | 50 |
| streptomycine | - | 500 |

Des stocks concentrés de chaque antibiotique sont conservés à -20°C. L'antibiotique est rajouté au milieu fondu, refroidi à 50°C, au moment de couler les boîtes.

3- Préparation et transformation de cellules compétentes d'E.coli.

3.1 - Préparation des cellules compétentes.

Une préculture d'une nuit de la souche d'E.coli est réalisée en milieu LB à partir d'une colonie isolée sur boîte. 500 ml de milieu LB sont inoculés avec 4 ml de cette préculture. Lorsque la densité optique à 600 nm atteint la valeur de 0,2, la culture est placée 15 à 20 min dans la glace puis centrifugée 10 min à 6000 rpm (rotor Kontron A8.24) à 4°C. Le culot est mis en suspension dans 200 ml de CaCl₂ 100 mM. Le culot est repris dans 5 ml de CaCl₂ 100 mM et placé dans la glace durant une nuit.

Les bactéries compétentes ainsi obtenues sont stockées en fractions de 1 ml en présence de glycérol à 30%. Elles peuvent ainsi se conserver plusieurs mois à -80°C.

3.2.- Transformation.

Les cellules compétentes sont décongelées ; 200 ml de suspension sont mis en présence de 0,1 à 1 µg

d'ADN pendant 30 min dans la glace . Le mélange est ensuite soumis à un choc thermique par passage de 2 min à 42°C. Après addition de 1 ml de LB liquide, le mélange est incubé pendant une heure à 37°C. L'ensemble
5 du milieu de transformation est mélangé à 3 ml de gélose molle maintenue à 45°C puis étalé sur milieu sélectif (milieu LB contenant les antibiotiques appropriés) . Les clones résistants sont subclonés et identifiés.

10 3.3- Identification des colonies bactériennes contenant les plasmides recombinants.

On réalise des minipréparations d'ADN plasmidique à partir d'un certain nombre de clones transformés . L'ADN de chaque clone est digéré par les
15 enzymes de restriction appropriées . Les fragments obtenus sont analysés par électrophorèse sur gel d'agarose, par rapport à un vecteur témoin. Ceci permet d'identifier les clones bactériens qui contiennent le plasmide recombinant.

20 4. Mobilisation E.coli - A.tumefasciens par conjugaison triparentale.

Le vecteur de transfert dans lequel est inséré le gène chimérique (plasmide Bin19) n'est pas directement transférable de E.coli à A.tumefasciens
25 mais peut être mobilisé si les fonctions de transfert et de mobilisation sont apportées par un plasmide dit "mobilisateur ". Il s'agit ici du plasmide pRK2013. Ce plasmide porte les fonctions de transfert du plasmide RK2 , le gène mob du plasmide ColE1 et l'origine de
30 réplication de ce même plasmide qui ne lui permet pas de se répliquer dans A.tumefasciens .

Les conjugaisons triparentales mettant en présence la souche d'E.coli donatrice, la souche d'A.tumefasciens réceptrice et la souche d'E.coli

possédant le plasmide pRK2013, sont réalisées.

Chaque souche est cultivée dans 5 ml du milieu de culture adéquat contenant les antibiotiques appropriés, pendant une nuit . Les cultures sont
5 centrifugées 5 min à 6000 rpm (Kontron A8.24) à 4°C . Les culots sont remis en suspension dans du milieu sans antibiotique puis centrifugés à nouveau . Chaque culot est finalement repris par 5 ml de milieu de culture.

On dépose ensuite 1 ml de chacune des
10 suspensions sur un filtre de porosité 0,45 µm (Millipore, type HA). Le filtre est placé sur milieu AP gélosé sans antibiotique dans une boîte de Pétri de diamètre 55 mm et incubé une nuit à 28°C.

Les bactéries sont récupérées sur le filtre et
15 remises en suspension dans 0,5 ml de milieu AP. Des dilutions de cette suspension sont effectuées et 150 à 200 µl de chaque dilution sont étalés sur milieu AP sélectif . Les boîtes sont placées à 28°C . Au bout de 3 à 5 jours , les clones apparaissent .

20 5- Extraction des acides nucléiques.

5.1 - Extraction de plasmides d'E.coli

a- minipréparation.

Le protocole suivant a donné les meilleurs résultats, de façon reproductible.

25 1,5 ml de milieu LB contenant les antibiotiques appropriés sontensemencés à partir d'une colonie dans un tube à essai. Le tube est ensuite placé à 37°C, sous agitation, durant une nuit. La culture est transférée dans un tube Eppendorf et centrifugée . Le culot est
30 repris par 150µl de STETL (saccharose 8%, Triton X100 5%, EDTA 50 mM, Tris 50 mM pH8, Lysozyme 0,5 mg/l). Le mélange est placé dans un bain-marie bouillant pendant 1 min, puis centrifugé 15 min. L'ADN chromosomique dénaturé , les protéines et les débris cellulaires

constituent un culot qui est éliminé. L'ADN du surnageant est précipité par addition de 150 μ l d'isopropanol pendant 15 min à -20°C. Après centrifugation, le culot est séché puis repris par 120 μ l de TE 10/1 (Tris 10mM, EDTA 1mM pH 7,5). Deux extractions au phénol-chloroforme (3v:1v) sont effectuées. L'ADN contenu dans la phase aqueuse est précipité par addition d'un dixième de volume d'acétate de sodium 3M et de deux volumes d'éthanol absolu. Après lavage à l'éthanol 80%, le culot est séché puis repris par 20 μ l de TE 10/1. 5 à 10 μ l de l'ADN préparé sont suffisants pour une analyse éventuelle, la quantité d'ADN pouvant varier en fonction de la taille du plasmide et de la souche bactérienne.

b- maxipréparation.

Après amplification au chloramphénicol (MANIATIS-Molecular Cloning - A Laboratory Manual Cold Harbor Laboratory, Cold Spring Harbor, New York, 1982), une culture bactérienne réalisée dans un litre de milieu LB liquide contenant les antibiotiques appropriés est centrifugée à 6000 rpm pendant 10 min (rotor Kontron A6.14), à 4°C. Les cellules récoltées sont lavées dans du TE 10/1 pH 8. Le culot est repris par 11,4 ml de TE 200/100 (Tris 200 mM, EDTA 100 mM pH 8) additionné de 0,6 ml de lysozyme à 20 mg.ml. Le mélange est laissé à température ambiante pendant 10 min. 4 ml de TE 200/100 pH 8 contenant 6% (p/v) de sarkosyl sont ajoutés et le tube est soumis à une agitation douce pendant 10 min à température ambiante. Les débris cellulaires sont éliminés par centrifugation à 12000 rpm pendant 30 min. Pour 7,6 ml de lysat récupéré, on ajoute 8 g de chlorure de césium (CsCl) et 1 ml de bromure d'éthidium (BET) à 10 mg/ml. Une ultracentrifugation est ensuite réalisée pendant 36

heures à 42000 rpm (rotor Beckman 50 Ti), à 20°C . A l'issue de cette centrifugation, les tubes sont éclairés en lumière UV (366 nm). Deux bandes fluorescentes sont observées :

- 5 - une bande supérieure constituée d'ADN chromosomique et d'ADN plasmidique ouvert,
- une bande inférieure constituée d'ADN plasmidique fermé (superenroulé).

10 La bande inférieure est récupérée soit à l'aide d'une seringue munie d'une aiguille, soit à l'aide d'une pompe péristaltique munie d'un cathéter . Le BET est extrait avec de l'isobutanol saturé en TE 10/1 pH 7,5. Une dialyse est ensuite réalisée contre du TE 10/1 pH 7,5 pendant environ 24 heures . Finalement, l'ADN
15 plasmidique est précipité par addition d'un dixième de volume d'acétate de sodium 3M et de deux volumes d'éthanol absolu. La concentration de la solution est estimée par spectrophotométrie.

5.2 - Extraction de l'ADN végétal total.

20 La méthode employée est décrite par ROGERS et BENDICH (Plant Mol. Biol. 5,1985, 69-76). Elle est basée sur l'utilisation de bromure de cétyltriméthylammonium (CTAB). Cette technique permet d'obtenir, chez le tabac, environ 20 µg d'ADN en
25 partant de 500 mg de feuilles .

 500 mg de feuilles jeunes sont broyées dans un mortier avec de l'azote liquide . Le broyat est transféré dans un tube Eppendorf . Un volume de 2 X CTAB à 65°C [CTAB 2% (p/v), Tris 100 mM pH 8, EDTA 20
30 mM pH 8, NaCl 1,4 M, PVP 1% (p/v)] est ajouté au broyat. Le mélange est incubé 5 min à 65°C et reçoit ensuite un volume de chloroforme-alcool isoamylique (24 v : 1v). Après homogénéisation et centrifuga- tion 5 min à 11000 rpm, la phase supérieure est récupérée et

transférée dans un nouveau tube . On ajoute alors un dixième de volume de CTAB 10% à 65°C [CTAB 10% (p/v), NaCl 0,7 M] , on homogénéise et on centrifuge de nouveau. La phase supérieure est récupérée et
5 additionnée d'un volume de tampon CTAB de précipitation à 65°C [CTAB 1 % (p/v), Tris 50 mM pH 8, EDTA 10 mM pH 8] . Après homogénéisation et centrifugation, le culot contenant les acides nucléiques est repris par 300 µl de TE high (Tris 10 mM pH 8, EDTA 1 mM pH 8, NaCl 1M)
10 et maintenu à 65°C pendant 10 min. L'ADN est précipité par addition de 600 µl d'éthanol absolu à -20°C pendant une nuit. Le lendemain, le culot est lavé dans l'éthanol à 80%, séché et repris par 20 µl de 0,1 X TE (Tris 1 mM pH 8, EDTA 1 mM pH 8).

15 6. Digestion de l'ADN par des endonucléases de restriction.

Les enzymes de restriction sont utilisées conformément aux indications données par le fournisseur (GIBCO). La composition du tampon d'incubation est
20 fonction de l'enzyme utilisée de même que la température d'incubation (pour la plupart des enzymes, cette température est de 37°C).

Le nombre d'unités enzymatiques utilisées dépend de l'ADN à digérer et de l'enzyme utilisée . Des
25 incubations de 1 à 2 heures avec 5 à 10 unités d'enzyme par µg d'ADN, pour l'ADN plasmidique et d'une nuit, pour l'ADN génomique sont généralement conseillées . Les ADNs obtenus par minipréparation sont traités par la RNase A préparée selon MANIATIS (1982 précédemment
30 cité) . Les digestions sont arrêtées par un passage de 10 min à 65°C.

7. Electrophorèse horizontale de l'ADN en gel d'agarose .

On utilise des gels d'agarose dont la

concentration varie de 0,8 à 3 % (p/v) , selon la
taille des fragments à séparer . L'agarose (Appligène,
qualité biologie molé- culaire) est mélangé au volume
requis de tampon TAE (Tris-acétate 0,04 M, EDTA 0,002
5 M pH 8) et porté à ébullition au four à microondes . Il
est refroidi jusqu'à 60°C avant d'être coulé dans la
cuve d'électrophorèse.

Lorsque les digestions ont eu lieu dans un
volume supérieur à 20 µl, l'ADN est précipité et le
culot, après rinçage à l'éthanol 80% , est repris dans
10 15 µl de TE 10/1 pH 7,5 . Les échantillons sont
additionnés d'un mélange [50% glycérol, EDTA 0,1 M,
Bleu de bromophénol 0,1 % , xylène cyanol 0,1%] pour
environ un cinquième de leur volume . Ceci permet de
15 suivre la migration.

La migration s'effectue à 20°C de la cathode
vers l'anode , sous une tension constante de 2 à 10
V/cm . La coloration des gels au BET (10 mg/ml) est
effectuée en même temps que la migration. Le gel est
20 observé et photographié sous lumière UV (254 nm). Les
masses moléculaires des fragments d'ADN sont estimées
par comparaison avec les fragments de l'ADN du
bactériophage lambda digéré par Hind III (Boehringer).

8. Elution d'un fragment d'ADN d'un gel
25 d'agarose.

Après électrophorèse, la purification de
fragments d'ADN peut être réalisée en découpant sous
UV, la bande d'agarose contenant le fragment à
purifier. Deux techniques d'élution ont été utilisées.

30 La première technique utilisée consiste en une
électroélution de l'ADN à l'aide d'un Biotrap (
commercialisé par Schleicher et Schuell). La bande
intéressante est placée dans le compartiment central du
Biotrap rempli auparavant de tampon TAE.

L'électroélution se déroule sous une différence de potentiel de 150 V durant 1 à 2 heures et peut être suivie en lumière UV. Après une inversion du courant de 20 secondes, la solution d'ADN peut être récupérée au niveau du réservoir délimité par deux membranes dont une retient l'ADN. Cette inversion du courant permet de libérer l'ADN retenu par la membrane. L'ADN est ensuite précipité à -20°C pendant 30 min après addition de 2 volumes d'éthanol absolu et d'un dixième de volume d'acétate de sodium 3 M. Le précipité est récupéré par centrifugation à 12000 rpm pendant 15 min, rincé avec de l'éthanol 80%. Le culot est ensuite séché et remis en suspension dans du TE 10/1 pH 7,5.

La seconde technique utilisée est celle du "freeze-squeeze" amélioré (TAUTZ et RENZ, Anal. Biochem. 132, 14-19 (1983)). Les parties du gel renfermant les bandes intéressantes sont découpées et équilibrées pendant 30 min à l'obscurité à 4°C contre 10 volumes de tampon TEN (Tris 10 mM pH 7,1, EDTA 1 mM, NaCl 0,3 M). Chaque fragment de gel est alors transféré dans un tube Eppendorf percé au fond et obstrué par de la laine de verre siliconée. Ce tube est lui-même introduit dans un autre tube Eppendorf. Le tout est congelé à -80°C, puis centrifugé 15 min à 12000 rpm. L'ADN est ensuite précipité et traité comme précédemment. Cette technique est beaucoup plus rapide que la technique d'électroélution utilisant le Biotrap et permet d'obtenir un rendement supérieur.

9. Ligatures des fragments de restriction par l'ADN ligase T4.

Les ligatures sont réalisées dans un volume final de 20 µl, à une température de 7 à 12 °C pendant toute la nuit à l'aide de l'ADN ligase du bactériophage T₄ (BRL). La composition du tampon de ligation est la

suivante :

- | | | |
|---|---------------------|--------------|
| | - Tris HCl | 50 mM pH 7,6 |
| | - MgCl ₂ | 10 mM |
| | - ATP | 1 mM |
| 5 | - DTT | 1 mM |
| | - PEG-8000 | 5 % (p/v) |

Les quantités relatives de vecteur et de fragment à insérer sont calculées de façon à avoir un excès de fragment à cloner d'un facteur 2. L'efficacité de la ligature peut être contrôlée par électrophorèse en gel d'agarose .

10. Transfert d'ADN sur membrane de nitrocellulose.

Après électrophorèse sur gel d'agarose, l'ADN est transféré sur une membrane de nitrocellulose (Hybond-C extra, Amersham) selon la technique décrite par SOUTHERN (J. Mol. Biol. 98, 503-517, 1975).

Après migration, le gel est photographié sous lumière UV (254 nm) puis baigné successivement 1 heure dans un bain de dénaturation (NaOH 0,5 M, NaCl 1,5 M) puis une heure dans un bain de neutralisation (Tris 0,5 M, NaCl 1,5 M).

Le transfert s'effectue durant la nuit dans du 10 X SSC préparé à partir d'une solution stock 20X SSC (NaCl 3 M, tricitrate de sodium 0,3 M pH 7).

L'ADN qui a été transféré est fixé définitivement sur la membrane par séchage sous vide à 80°C pendant 2 heures.

11. Marquage radioactif d'une sonde moléculaire par extension d'amorce au hasard (" random priming").

11. 1- Principe

Cette méthode a été développée par FEINBERG et VOGELSTEIN (Anal. Biochem. 132, 6, 1983; Anal. Biochem. 137, 266, 1984) . Le marquage du fragment

choisi comme sonde est réalisé par amorçage au hasard suivi d'une extension à l'aide du fragment de Klenow de l'ADN polymérase d'E.coli.

5 L'ADN dénaturé est incubé dans un milieu contenant un mélange d'hexanucléotides, les quatre désoxynucléotides froids et du $[\text{gamma } ^{-32} \text{P}] \text{ dCTP}$. L'action du fragment de Klenow de l'ADN polymérase I conduit à la synthèse d'une sonde radioactive dont l'activité spécifique se situe entre 2 et $5 \cdot 10^8$ cpm par
10 μg d'ADN.

11.2 - Aspects pratiques.

L'ADN à marquer (25 à 100 ng) est dénaturé par la chaleur (100°C pendant 5 min), le tube est ensuite placé à 4°C . Le milieu réactionnel suivant est ajouté:

| | | |
|----|--|---------------------------|
| 15 | - Mg Cl_2 | 1,2 mM |
| | - β -mercaptoéthanol | 2,5 mM |
| | - Tris-HCl pH 8 | 1,2 mM |
| | - Hepes pH 6,6 | 50 mM |
| | - d NTP | 10 mM |
| 20 | - $[\text{gamma } ^{-32}\text{P}] \text{ d CTP } 10 \text{ mCi.ml}^{-1}$ | 50 μCi |
| | - Hexamères | 0,3 UDO _{260 nm} |
| | - Fragment de Klenow | 4 U |
| | - BSA | 0,4 mg.ml^{-1} |
| | - Eau distillée q.s.p. | 50 μl |

25 L'ensemble est incubé pendant environ 1 heure à 37°C. La réaction est arrêtée par addition d'un dixième de volume de d'EDTA 0,5 M.

12. Hybridation moléculaire et autoradiographie

30 La membrane de nitrocellulose portant l'ADN transféré et fixé est soumise à une étape de préhybridation à 68°C pour une durée de 1 à 2 heures, dans environ 10 ml de la solution suivante :

- 6X SSC
- 5X Denharts préparé à partir d'une solution

100X:

BSA 2% (p/v)
Ficoll 2% (p/v)
PVP 2% (p/v)
5 - SDS 0,5 % (p/v)

-0,1 mg.ml⁻¹ d'ADN , dénaturé et soumis aux
ultra-sons, de sperme de saumon.

On ajoute ensuite à cette solution, la sonde
auparavant marquée et dénaturée par la chaleur .
10 L'hybridation s'effectue à 68°C pendant une nuit. La
préhybridation et l'hybridation sont réalisées dans un
sac de polyéthylène hermétiquement soudé.

La membrane est ensuite lavée dans les bains
suivants :

15 - deux fois 15 min dans du 2X SSC à 65°C
- 30 min dans du 2X SSC; 0,1 % SDS à 65°C. Cette
dernière étape peut être répétée si nécessaire.

Après séchage, la membrane est enveloppée dans
un film plastique étirable, puis placée dans une
20 cassette d'auto-radiographie avec un film Kodak X-ray
inséré entre deux écrans intensificateurs. Cette
cassette est alors mise à -80°C . La durée de
l'exposition peut varier de quelques heures à une
semaine selon l'intensité du signal.

25 13. Réaction de polymérisation en chaîne (PCR)

13.1- Principe

Le principe de la technique de PCR repose sur
l'utilisation d'un couple d'amorces (" primers"),
oligonucléotides de synthèse qui délimitent la région à
30 amplifier sur l'ADN matriciel . L'un des deux
oligonucléotides est complémentaire d'une partie du
brin sens, l'autre oligonucléotide étant complémentaire
d'une partie du brin anti sens . Chaque cycle de PCR
comprend trois étapes successives :

- dénaturation de l'ADN
- hybridation des amorces sur l'ADN matriciel
- élongation des chaînes complémentaires de l'ADN par la Taq ADN polymérase depuis les extrémités 3'OH.

L'obtention d'une quantité notable d'ADN amplifié nécessite d'effectuer un nombre important de cycles (entre 25 et 40) . L'efficacité de l'amplification est de l'ordre de 10^7 , ce qui permet d'obtenir au bout de 30 cycles , environ $1\mu\text{g}$ d'un fragment d'ADN de 300 pb, à partir d'un μg d'ADN génomique .

13.2 - Aspects pratiques

a- Synthèse des oligonucléotides

La synthèse chimique des deux oligonucléotides utilisés est réalisée sur l'appareil Gene Assembler Plus (Pharmacia LKB) . Cet appareil utilise des dérivés de nucléotides de type β -cyanoéthylphosphoramide . Il permet la synthèse de $0,2\mu\text{mole}$ d'oligonucléotides avec une efficacité de couplage supérieure à 90% ne nécessitant pas une purification ultérieure des oligonucléotides dans la mesure où ils n'excèdent pas une longueur de 30 bases.

b - Réaction de PCR

Les réactions de PCR sont réalisées sur l'automate Gene ATAQ Controller (Pharmacia LKB) . Elles sont effectuées dans des volumes de $50\mu\text{l}$ contenant :

- 50 pmoles de chaque amorce
- $1\mu\text{g}$ d'ADN génomique
- dATP, dCTP, dGTP, dTTP : 2 mM chacun

- tampon de PCR (Proméga) :

Tris-HCl 10 mM pH 8,4

KCl 50 mM

MgCl₂ 1,5 mM

5 Gélatine 0,1 mg.ml⁻¹

Triton X100

- 1,25 U de Taq ADN polymérase (Proméga)

L'ADN génomique est préalablement dénaturé à 100°C pendant 5 min en présence des amorces et de 50 µl d'huile minérale pour éviter toute évaporation, puis placé immédiatement dans la glace . L'eau , les désoxynucléotides , le tampon de PCR et la Taq ADN polymérase sont déposés sur l'huile . Une brève centrifugation est nécessaire pour permettre à ces derniers composants de traverser l'huile . Après une étape d'élongation à 72°C, 30 cycles de PCR sont effectués, comprenant chacun :

- une étape de dénaturation de 30 sec à 94°C

20 - une étape d'hybridation de 30 sec à la température choisie

- une étape d'élongation de 2 min à 72°C.

Les produits de PCR sont séparés par électrophorèse sur gel d'agarose.

II. TECHNIQUES RELATIVES A LA BIOLOGIE VEGETALE

25 1 - Milieux de culture

1.1- Milieu de base.

Le milieu de base est dérivé du milieu de MURASHIGE et SKOOG (Physiol. Plant, 15, 473-497, 1962). A partir de ce milieu , des modifications permettent d'obtenir un certain nombre de milieux aux propriétés différentes suivant l'utilisation souhaitée. Sa composition est indiquée dans le tableau ci-dessous:

Composition du milieu de base

| Composition du milieu de base | | | | |
|-------------------------------|-------------------------------------|------------------------|---|-------|
| Macroéléments (mg/l) | | Microéléments (mg/l) | | |
| 5 | KNO ₃ | 1900 | MnSO ₄ 4H ₂ O | 22,3 |
| | NH ₄ NO ₃ | 1650 | ZnSO ₄ 7H ₂ O | 8,6 |
| | CaCl ₂ 2H ₂ O | 440 | H ₃ BO ₃ | 6,2 |
| | MgSO ₄ 7H ₂ O | 370 | KI | 0,83 |
| | KH ₂ PO ₄ | 170 | Na ₂ MO ₄ 2H ₂ O | 0,25 |
| 10 | | | COCl ₂ 6H ₂ O | 0,025 |
| | | | CuSO ₄ 5H ₂ O | 0,025 |
| | | | FeSO ₄ 7H ₂ O | 27,85 |
| | | | Na ₂ EDTA | 37,25 |
| Vitamines (mg/l) | | | | |
| 15 | Inositol | 100 | pyridoxine HCl | 0,5 |
| | Thiamine HCl | 0,1 | acide nicotinique | 0,5 |
| Saccharose 20 g/l | | | | |

1.2 - milieu I.

Il s'agit du milieu de base contenant en plus de l'acide naphthalène acétique (ANA) à 0,2 mg/l et de la benzylaminopurine (6-BAP) à 1 mg/l . Il est utilisé pour la mise en culture des disques foliaires, après inoculation par A.tumefasciens.

1.3 - milieu II.

Ce milieu correspond au milieu I additionné de céfotaxime à 200 mg/l , antibiotique destiné à bloquer la croissance des agrobactéries, et de kanamycine à 50 mg/l . Il permet le développement et la sélection de colonies cellulaires pouvant générer la formation de bourgeons puis de plantes transformées .

1.4 - milieu III

Ce milieu est obtenu par addition de kanamycine (50 mg/l) au milieu de base . C'est un milieu de repiquage , où l'absence de régulateurs de croissance permet d'induire le développement de racines à partir des plantules régénérées sur milieu II.

1.5 - milieu IV.

Il s'agit du milieu II comportant, comme seul antibiotique, de la kanamycine (50 mg.l⁻¹) . Il est utilisé pour les tests de résistance à la kanamycine sur fragments de feuilles .

Tous les milieux décrits ici sont des milieux solides qui contiennent 0,7 % d'agar (qualité " plant tissue culture ", commercialisé par SIGMA) . Le pH est ajusté à 5,7 avant stérilisation (115°C pendant 20 min).

2. Matériel végétal

2-1 - Choix du matériel végétal

Le choix du tabac (Nicotiana tabacum cv Paraguay Bell) en tant que source d'explants utilisables dans nos expériences de transformation a été guidé par un souci de facilité technique , la notoriété de l'espèce , en matière de culture in vitro, n'étant plus à établir . Il s'agit d'une variété tétraploïde .

2-2 - Désinfection des feuilles

Les feuilles destinées aux expériences d'inoculation sont prélevées sur des plants de tabac cultivés en condition non aseptiques . Une désinfection préalable est donc indispensable . Ceci est réalisé en immergeant les feuilles dans une solution d'hypochlorite de calcium à 1 % pendant 20 min. Trois rinçages à l'eau distillée stérile sont ensuite effectués .

3 - Technique d'inoculation de disques foliaires.

3.1- Choix et principe de la technique.

Le principe de cette technique est simple : des
5 fragments de feuilles sont mis en contact avec les
agrobactéries puis transférés sur un milieu induisant
la formation de bourgeons et contenant l'agent
sélectif. En différents points du bord des fragments,
apparaissent des bourgeons dont l'isolement est aisé.
10 Pour obtenir des plantes résultant d'évènements de
transformation indépendants , il est nécessaire
d'isoler des bourgeons de fragments différents ou de
centres de régénération éloignés sur un même fragment .
Comparée à la technique de co-culture de protoplastes,
15 cette méthode présente l'avantage de réduire la phase
de croissance du tissu indifférencié . Ainsi , on peut
réduire l'apparition d'aberrations génétiques induites
par la culture in vitro . De plus , grâce à cette
technique, la régénération de plantes est plus rapide
20 et les fragments foliaires sont en outre beaucoup moins
délicats à manipuler que les protoplastes.

3.2 - Aspects pratiques.

Après désinfection des feuilles, la nervure
principale, les nervures latérales importantes ainsi
25 que les bords des limbes sont éliminés . Les feuilles
sont ensuite découpées en fragments d'environ 1 cm².
Les fragments sont déposés , face inférieure vers le
haut, à la surface d'une suspension d'agrobactéries
obtenue par dilution au dixième d'une culture de la
30 nuit auparavant lavée dans de l'eau distillée stérile .
Des témoins de non inoculation sont réalisés en
incubant des fragments foliaires dans de l'eau
distillée stérile .

Après 15 minutes d'incubation, les fragments

sont égouttés sur du papier filtre stérile et transférés , à raison de 5 fragments par boîte de Pétri de 90 cm de diamètre, sur milieu I.

5 Au bout de 48 heures , les fragments sont prélevés pour être transférés sur milieu II. Des repiquages sur ce même milieu sont ensuite effectués de façon régulière (toutes les trois semaines) afin de maintenir l'efficacité de l'agent sélectif .

10 4- Préparation d'échantillons en vue d'observations en microscopie photonique et électronique.

15 Des fragments d'environ 1 mm sur 2 mm sont prélevés à des niveaux comparables , sur des feuilles de même rang. Ces échantillons sont fixés, post-fixés et déshydratés selon le procédé décrit précédemment (matériel et méthodes , première partie) . Les inclusions sont réalisées dans un mélange EPON-ARALDITE.

20 Les coupes semi-fines , destinées aux observations en microscopie photonique, sont colorées au bleu de toluidine. Les coupes ultrafines, destinées aux observations en microscopie électronique, sont contrastées à l'acétate d'uranyle et au citrate de plomb.

25 5. Dosage de la chlorophylle.

La technique de dosage utilisée dérive de la méthode décrite par HOLDEN (Chlorophylls in : Goodwin T.W. -Chemistry and Biochemistry of Plants Pigments ed: Academic Press, 1965, 462-488.)

30 150 mg de feuilles (poids frais) sont broyées, à 4°C, dans un mortier en présence de 1 ml d'acétone à 80%. L'extrait acétonique est recueilli par décantation et conservé au froid , à l'abri de la lumière . Les résidus sont broyés dans 5 ml d'acétone à 80% .

L'extrait acétonique obtenu à l'issu de ce second broyage est récupéré par décantation. Cette opération est renouvelée le nombre de fois nécessaire pour que l'extrait acétonique, lors du dernier broyage soit incolore . Les solutions acétoniques sont rassemblées et ajoutées au broyat provenant de la dernière extraction. Après une centrifugation de 10 min à 4500 rpm (rotor Kontron A 8.24) à 4°C, le surnageant est transféré dans une fiole jaugée afin de déterminer le volume de l'extrait acétonique total. Le volume de l'extrait acétonique total est complété à 25 ml.

Le dosage des chlorophylles est basé sur l'absorption de la lumière par les extraits chlorophylliens dans l'acétone à 80% . La concentration en chlorophylle du surnageant acétonique est estimée par mesure de la DO à 652 nm. Elle est ensuite ramenée au poids sec de feuilles et exprimée en mg de chlorophylle par g de feuilles (poids lyophilisé).

6. Détermination des échanges gazeux respiratoires à l'aide de l'oxygraphe.

6.1 - Principe.

L'oxygraphe permet la mesure de la concentration en oxygène d'un milieu liquide . L'appareillage comprend :

- une anode d'argent et une cathode de platine reliées par un pont de chlorure de potassium et séparées d'une cellule de mesure par une membrane de TEFLON perméable à l'oxygène

- un boîtier de polarisation de l'électrode de mesure,

- un potentiomètre enregistreur.

Le principe de la méthode consiste à mesurer le courant électrique qui provient de la réaction d'oxydoréduction faisant intervenir l'oxygène dissous ,

l'électrode polarisée (ici la cathode de platine) et la réaction d'oxydation d'une anode d'argent . La tension de polarisation est maintenue constante tout au long de l'expérimentation .

5 6.2 - Aspects pratiques .

Pour chaque expérience, l'électrode est calibrée à 0% et à 100% d'oxygène . La valeur de 0% est obtenue par addition, dans le milieu , de cristaux d'hydrosulfite de sodium. La valeur de 100% est obtenue
10 avec de l'eau saturée en oxygène , à la même température que le milieu expérimental.

Les mesures sont réalisées à l'obscurité en milieu agité , sur des fragments de feuilles d'environ 50 mg (poids frais). Les fragments sont introduits
15 dans la chambre de mesure auparavant remplie d'une solution tampon constituée du mélange carbonate de sodium 0,1 M/bicarbonate de sodium 1 M (lv: 19 v). On enregistre les variations de la concentration en oxygène due à la respiration des tissus dans le milieu réactionnel, pendant un temps donné . La quantité
20 d'oxygène consommée par les tissus , exprimée en μ moles d'O₂ par mn et par mg de feuilles (poids lyophilisé) peut ainsi être déterminée par rapport à la valeur 100% d'oxygène (concentration maximale en
25 oxygène d'une solution saturée d'air, à la température de l'expérience).

EXEMPLE 1

Clonage du gène ERG12 dans un vecteur de transfert mobilisable vers A.Tumefasciens.

30 1 - Clonage du gène ERG12 dans le vecteur de transfert.

La carte de restriction partielle du fragment Bam H I-Bam H I de 2,8 kb qui contient le gène ERG12 codant pour la MK est représenté figure 3 . Ce fragment

de 2,8 kb a été cloné dans le plasmide pFL44 au niveau du site de restriction Bam H I. Le vecteur ainsi obtenu est le pAO11.

5 La première étape de la construction a consisté à cloner le gène ERG12 dans le vecteur pLBR 19 représenté sur la figure 4 et qui comprend une cassette d'expression constituée du promoteur (présent en deux exemplaires disposés en tandem) et de la séquence de polyadénylation de l'ARN 35S du virus de la mosaïque du
10 chou-fleur (CAMV). Un site de clonage multiple a été inséré entre ces deux séquences . Cette région, d'une taille voisine de 1,5 kb, est délimitée par les sites uniques de restriction Sac I et Xho I. Ce vecteur porte en plus une origine de replication fonctionnelle dans
15 E.coli et un marqueur permettant la sélection dans E.coli.

La carte de restriction partielle du plasmide pFL44 (figure 5), fait apparaître que le fragment Bam H I-BamH I de 2,8 kb contenant le gène ERG12, est bordé
20 par les sites uniques de restriction Pst I et Sma I. Le site multiple de clonage du plasmide pLBR19 représenté sur la figure 4 comporte également ces deux séquences de reconnaissance . Le fragment Pst I-Sma I comprenant l'insert BamH I-BamH I de 2,8 kb, a donc été isolé de
25 pAO11, séparé sur gel d'agarose et cloné, après élution, dans pLBR19 (figure 6). La construction ainsi obtenue (pFAB1) a été utilisée pour transformer E. coli 71/18. Les clones contenant le plasmide recombinant ont été sélectionnés sur milieu LB contenant de
30 l'ampicilline. L'ADN plasmidique extrait des colonies sélectionnées a été hydrolysé par BamH I et analysé par électrophorèse sur gel d'agarose à 0,8%. Le profil de restriction obtenu (figure 7, piste 2) a été comparé à celui du plasmide pAO11 hydrolysé par la même enzyme :

deux bandes caractéristiques , l'une correspondant à un fragment de 1,8 kb et l'autre correspondant à un fragment de 0,8 kb , sont observées . Les tailles de ces fragments sont celles attendues en comparaison du profil de restriction provenant de l'hydrolyse de pAO11 par BamH I (figure 7 piste 1).

Afin d'orienter correctement l'insert par rapport au promoteur 35S du CAMV, on a choisi de cloner l'ensemble du fragment [35S-ERG12-3' 35S], délimité par les sites de restriction Xho I et Sac I et d'une taille voisine de 4,3 kb, dans le vecteur de transfert Bin19 .

La carte de restriction partielle du vecteur de transfert Bin19 est représentée figure 8. L'information génétique que l'on souhaite introduire dans le génome végétal est clonée entre les frontières RB et LB portées par ce plasmide . Ce dernier est introduit dans la souche LBA4404 d'*A.tumefasciens* hébergeant un plasmide Ti (pTi) dépourvu de la région T. Le transfert de la séquence clonée est alors permis grâce aux fonctions de virulence portées par ce pTi. Le vecteur de transfert Bin19, utilisé dans des systèmes binaires de transformation est compatible avec les plasmides portant les fonctions de virulence et possède :

- une origine de replication fonctionnelle dans *E.coli*,
- une origine de replication fonctionnelle dans *A.tumefasciens*,
- un marqueur de sélection bactérien (gène codant pour la résistance à la kanamycine),
- un gène chimérique de résistance à la kanamycine permettant la sélection des cellules végétales transformées . Ce gène est inséré entre les séquences bordures droite et gauche du plasmide et est

co-transféré dans le génome des cellules végétales.

Le fragment XhoI- Sac I comportant le gène ERG12 a été isolé du vecteur pFAB1 et séparé sur gel d'agarose . Après élution, il a été cloné dans le vecteur de transfert au niveau des sites Sac I et Sal I qui génèrent des extrémités compatibles avec celles provenant d'une hydrolyse par Xho (figure 9) . La construction résultant de ce clonage (pFAB2) a été introduite, par transformation dans E.coli HB101 et les clones contenant le plasmide recombinant ont été sélectionnés sur milieu LB contenant de la kanamycine . Comme précédemment, l'ADN plasmidique extrait des colonies sélectionnées a été hydrolysé par BamH I et analysé par électrophorèse sur gel d'agarose à 0,8 % . Les bandes caractéristiques (1,8 kb et 0,8 kb) ont été retrouvées (figure 10, piste 3) .

2. Introduction du vecteur de transfert pFAB2 portant le gène ERG12 dans A.tumefasciens.

Le vecteur pFAB2 a été introduit par conjugaison dans la souche LBA4404 d'A.tumefasciens, résistante à la streptomycine et à la rifampicine en présence d'une souche "helper" d'E.coli comportant le plasmide pRK2013 porteur d'un gène de résistance à la kanamycine . Ce plasmide, incapable de se répliquer dans A.tumefasciens est perdu , à l'inverse du vecteur de transfert pFAB2 qui lui, est conservé et peut être amplifié. Seuls les clones d'A.tumefasciens qui ont reçu ce vecteur seront capables de se développer sur un milieu AP contenant de la kanamycine, de la streptomycine et de la rifampicine.

Lors des expériences de conjugaison, des colonies résistantes à ces trois antibiotiques ont été obtenues aussi bien en présence qu'en absence du plasmide "helper" . Les colonies ainsi sélectionnées

ont été cultivées en milieu AP liquide contenant de la kanamycine et celles qui ont été capables de croître sur ce milieu ont été retenues pour les expériences d'inoculation de disques foliaires.

5 3 - Essais qualitatifs d'activité MK

 Ce test , mis au point par SERVOUSE et KARST (Biochem. J., 240; 541-547 , 1986) permet de contrôler de façon simple et rapide la présence ou l'absence d'une activité MK dans les bactéries transformées par les différents plasmides recombinants.

10 Le contenu d'une petite anse de bactéries préincubées la veille sur milieu solide approprié , est mis en suspension dans du tampon phosphate 0,1 M pH 7,4. Une goutte de toluène est ajoutée afin de perméabiliser les cellules . Après une incubation à 15 37°C pendant 30 min, les cellules sont mises en contact avec le mélange réactionnel (ATP 20 mM, MgCl₂ 10 mM, [¹⁴C] AMV 1,5 mM) et incubées 15 à 30 min à 35°C. La réaction est bloquée par chauffage à 100°C pendant 5 20 min. Après centrifugation, le surnageant est analysé par chromatographie sur couche mince de gel de silice dans le système n-propanol-ammoniac-eau (6v: 3v: 1v). La distribution de la radioactivité, déterminée par lecture de la plaque avec un scanner de radioactivité 25 Berthold 2832 automatic TLC linear Analyser, permet de visualiser la transformation de l'AMV en AMVP (figures 11 et 12).

 Ce test, effectué pour la souche d'E.coli porteuse du vecteur pFAB1 (figure 11C) ainsi que pour 30 la souche d'A.tumefasciens porteuse du vecteur pFAB2, a permis de déceler dans chaque cas , une activité MK . Les souches E.coli 71/18 [pFL44] (figure 11A) et E.coli Hb 101 [pBin 19] (figure 12A) constituent les témoins négatifs . La souche E.coli 71/18 [pAO11]

(figure 11B) constitue , quant à elle , le témoin positif.

EXEMPLE 2:

Transfert du gène et vérification de sa présence
5 dans le génome des plants de tabac régénérés.

1- Obtention des plantes transformées.

Des fragments de feuilles de plants de tabac ont été traités par la souche d'A.tumefasciens porteuse du vecteur pFAB2 selon la technique d'inoculation de
10 disques foliaires décrite dans le chapitre "matériel et méthodes ". Les fragments ont été placés sur milieu de régénération contenant de la kanamycine (milieu I). Des fragments témoins de résistance à la kanamycine (notés " témoins Kan^R") ont été mis en culture sur ce même
15 milieu après inoculation par la souche d'A.tumefasciens porteuse du plasmide Bin19. Enfin, des fragments témoins de régénération (notés " témoins H₂O") ont été cultivés sur milieu de régénération sans kanamycine après avoir été incubés dans de l'eau.

20 Une cinquantaine de plantes résultant d'évènements de transformation indépendants ont été transférées en serre. Elles proviennent de quatre expériences d'inoculation différentes . Ces plantes sont fertiles puisqu'elles ont fleuri et donné des
25 graines.

2- Test de résistance à la kanamycine.

Ce test consiste simplement à induire, sur milieu contenant de la kanamycine (milieu IV), la
formation de cals à partir d'un fragment foliaire . Il
30 est rapide et sans ambiguïté .

Des feuilles prélevées sur les plantes issues d'expériences de transformation et de plantes " témoins H₂O " sont découpées en fragments d'environ 1 cm² . Les fragments sont ensuite placés sur milieu IV.

Les fragments qui proviennent de plantes "témoins H₂O" blanchissent très rapidement lorsqu'ils sont cultivés en présence de kanamycine contrairement aux fragments issus de plantes transformées, à partir
5 desquelles, il est de nouveau possible d'induire une régénération.

3. Contrôle de la présence du gène .

3.1 - Southern blot

L'ADN génomique des plantes transformées a été
10 isolé selon la technique de ROGERS et BENDICH (Plant Mol. Biol. 5, 69-76 1985), décrite dans " matériel et méthodes " . Il a été hydrolysé par Bam HI, puis après électrophorèse, transféré sur membrane de nitrocellulose. Il a été ensuite hybridé avec une sonde
15 correspondant au fragment Bam HI-Bam HI de 1,8 kb qui contient probablement la plus grande partie du gène ERG12 (OULMOUDEN et KARST, Curr. Genet, 19, 1991, 9-14, 1990).

La figure 13 représente les résultats d'une
20 expérience d'hybridation: on retrouve, au niveau de la piste correspondant à l'ADN extrait d'une plante transformée (piste 3), un fragment dont la taille est celle que l'on pouvait attendre (1,8 kb), montrant ainsi que l'intégration du gène ERG12 a bien eu lieu.
25 Cette série d'hybridation comporte également deux témoins:

- un témoin correspondant à l'hydrolyse par Bam HI de l'ADN génomique d'une plante , " témoin H₂O", hybridé avec la même sonde (piste 2) : aucune bande
30 n'est observée,

- un témoin correspondant à l'hydrolyse par Bam HI de l'ADN du plasmide pAO11 (vecteur d'où a été isolé le gène ERG12 et qui constitue le témoin positif), hybridé avec la même sonde : on retrouve une bande

correspondant à un fragment d'une taille de 1,8 kb (piste 1).

Il convient cependant de noter que l'obtention d'une réponse claire et sans ambiguïté, par cette technique, nécessite, en particulier chez les végétaux, l'extraction d'une quantité relativement importante d'ADN. Ceci est à mettre en relation avec la taille importante des génomes végétaux et avec le faible nombre de copies généralement présentes dans le génome des plantes transformées par les agrobactéries.

3-2 - Réaction de polymérisation en chaîne (PCR).

a- choix des amorces

Les deux oligonucléotides (appelés ON₁ et ON₂) synthétisés chimiquement et utilisés comme amorce dans les réactions de PCR ont été choisis de façon à avoir à l'intérieur du fragment amplifié, un site unique reconnu par l'enzyme de restriction Bam HI. Leurs séquences sont les suivantes :

20 - ON₁ 5' ATGTCATTACCGTTCTTAACTTCT 3'
 - ON₂ 5' GCTTGAGCCCAACCC 3'

La localisation de ces deux oligonucléotides et celle du site Bam HI, au niveau de la séquence nucléotidique du gène ERG12 est représentée figure 14. La partie amplifiée in vitro doit correspondre à un fragment de 447 pb. Une hydrolyse par Bam HI du fragment amplifié doit libérer deux fragments de 150 et 297 pb.

b - résultat de l'amplification.

30 Le choix d'une température d'hybridation de 45°C a été guidé par la température de fusion des amorces (T_m) qui détermine l'hybridation de ces mêmes amorces sur l'ADN à amplifier.

Une bande d'une taille voisine de 447 pb est

amplifiée avec le couple d'amorces ON₁ et ON₂ pour les échantillons correspondant à l'ADN génomique des plantes transformées (figure 15, pistes 1 à 6) de même que pour l'échantillon d'ADN génomique de levure qui constitue le témoin positif (figure 15, piste 7). Les résultats qui concernent les différentes plantes transformées ont été obtenus après suramplification. Cette étape permet de mieux visualiser les bandes dont l'intensité était faible , dans certains cas, après la première amplification .

Aucune bande d'amplification n'est obtenue avec les amorces ON₁ et ON₂ seules, à partir de l'ADN génomique de levure (figure 15, pistes 8 et 9): la bande d'amplification visible au niveau des pistes 1 à 7 résulte donc bien d'une amplification obtenue avec les deux amorces.

Aucune bande d'amplification n'est observée dans le cas d'ADN génomique isolé de plante " témoin H₂O" (figure 15, piste 10).

Un autre contrôle consistant à remplacer l'ADN par de l'eau stérile prouve qu'aucun des constituants utilisés n'était contaminé par de l'ADN exogène puisqu'aucune bande d'amplification n'est observée dans ce cas (figure 15, piste 11).

c- analyse du fragment amplifié.

L'obtention d'une bande d'une taille voisine de 447 pb ne suffit pas pour affirmer qu'il s'agit bien d'une amplification à partir du gène ERG12. Le fragment amplifié a donc été élué après migration, hydrolysé par Bam HI et analysé par électrophorèse sur gel d'agarose. Deux bandes sont observées; l'une correspond à un fragment d'une taille voisine de 297 pb et l'autre à un fragment d'une taille voisine de 150 pb. Ce profil est obtenu aussi bien à partir d'ADN génomique de levure

(figure 16, piste 2) qu'à partir d'ADN génomique de plantes transformées (figure 16, piste 1). Le fragment amplifié, dans ce dernier cas, correspond donc bien au gène ERG12.

5 3.3- Conclusion.

L'ensemble des résultats obtenus par Southern blot et par PCR montre donc que l'intégration du gène ERG12 dans le génome des plantes transformées a bien eu lieu.

10 EXEMPLE 3 :

Etude des effets de l'insertion.

1) Effets sur la croissance et sur la régénération des plantes transformées.

1.1 Régénération

15 a- observations.

Deux semaines sont nécessaires pour voir apparaître, chez les "témoins H₂O", un début de callogenèse qui prend place tout d'abord sur le bord des fragments mis en culture et qui envahit ensuite progressivement la surface des explants ; au bout d'un mois, le cal est assez abondamment développé. A ce stade, la différenciation de racines précède très souvent l'organisation de bourgeons.

20 Chez les "témoins Kan^R", la callogenèse est faible. Il y a formation de bourgeons entre trois semaines et un mois après la mise en culture des explants. Cette différenciation semble pouvoir s'initier à partir de cals peu développés mais également de manière plus directe, en différents points
30 du pourtour du fragment.

Pour les fragments traités par la souche d'*A. tumescens* porteuse du vecteur pFAB2, la différenciation intervient sans que l'on puisse auparavant discerner la formation d'un cal. Des

bourgeons sont visibles, en différents points du bord des explants, trois semaines après leur mise en culture. Ceci est comparable à ce que l'on observe pour les fragments " témoins Kan^R" . Cependant, le développement ultérieur de ces bourgeons semble beaucoup plus rapide . Simultanément, une callogenèse qui semblait pratiquement inexistante au départ, prend place . A partir de ces groupes de cellules indifférenciées, peuvent se différencier de nouveaux centres organogènes qui donneront naissance à de nouveaux bourgeons. Ainsi, le nombre de centres de régénération, au niveau des fragments traités par la souche d'A.tumefasciens porteuse du vecteur pFAB2 est plus élevé que pour les "témoins H₂O" et "Kan^R". Il en résulte un nombre également plus important de plantules régénérées .

Les plantules en cours de régénération sont excisées et repiquées en boîte de Pétri sur milieu sans hormone mais contenant toujours l'agent sélectif (milieu III), afin de favoriser le développement de racines . Les plantules qui proviennent des fragments "témoins H₂O" sont, quant à elles, transférées sur milieu sans hormone et sans agent sélectif . Lorsque les racines apparaissent, les plantes sont repiquées sur le même milieu contenu dans des boîtes de culture Magenta GA₃ (Poly Labo) pour permettre leur développement . Quelques semaines après cette étape (en général deux semaines), une différence de croissance notable peut déjà être observée : les feuilles des plantes régénérées à partir d'explants inoculés par la souche d'A.tumefasciens porteuse du vecteur pFAB2 sont plus développées, les entrenoeuds sont plus courts et les tiges semblent beaucoup plus "trapues", ceci en comparaison des plantes " témoins H₂O" et "Kan^R" . Les

plantes " témoins Kan^R" ont une allure similaire aux plantes " témoins H₂O" . Deux mois après le transfert en boîte de culture Magenta, les plantes régénérées sont transférées en condition non aseptique .

5 Il faut donc environ quatre mois, à compter du jour de l'inoculation, pour obtenir des plantes complètes qui soient capables de croître après transfert en terre. Ces plantes sont ensuite maintenues sous cloche durant un mois au laboratoire sous
10 photopériode naturelle, afin de réduire au maximum les traumatismes provoqués par le sevrage. Elles sont, après cette période d'acclimatation, cultivées en serre.

b - Conclusion.

15 Comparés aux différents témoins, les processus intervenant dans la régénération des plantes ayant intégré dans leur génome le gène ERG12, se caractérisent par :

20 - une réduction plus ou moins importante de la phase cal au profit d'une organogenèse plus précoce se traduisant par une différenciation presque directe de bourgeons,

25 - un nombre de centres de régénération plus élevé consécutif à une différenciation s'initiant à partir de centres secondaires,

 - un développement des bourgeons beaucoup plus rapide,

 - des plantes d'aspect plus "trapu" avec des feuilles plus développées.

30 1.2 - Caractéristiques morphologiques et croissance des plantes transformées .

a. Observations.

Un suivi régulier des plantes transformées , cultivées en serre, a été effectué . Leur croissance et

leurs caractéristiques morphologiques ont été comparées uniquement à celle des plantes " témoin H₂O" .

5 Le phénomène de précocité observé lors de la régénération, au niveau de la différenciation des bourgeons, se maintient chez les plantes adultes . Cette précocité, très marquée durant la phase de croissance des plantes , s'atténue toutefois légèrement à mesure que l'on s'achemine vers la floraison. Dans la majeure partie des cas, la floraison des plantes ayant
10 intégré dans leur génome le gène ERG12 et celle des plantes témoins sont décalées dans le temps (de deux à trois semaines). Ce décalage est toujours favorable aux plantes transformées . Pour un cas particulier , un décalage allant dans le même sens mais , cette fois-ci,
15 beaucoup plus marqué (de l'ordre de trois mois) , a été observé sans que l'on puisse savoir si cela était une conséquence extrême de l'insertion du gène .

Le phénomène d'accélération de la croissance, observé initialement , lors du développement des
20 bourgeons , se maintient également chez les plantes adultes. Les plantes transformées ont une taille plus importante et un nombre de rangs plus élevé (accélération du plastochrone). Leurs entrenoeuds sont maintenant plus développés que chez les plantes
25 témoins. Ce phénomène s'atténue à mesure que l'on se rapproche de la floraison. La surface foliaire est augmentée (facteur variant de 1,3 à 1,5), en particulier pour les feuilles situées à la base de la plante.

30 Enfin, les plantes transformées montrent des inflorescences plus ramifiées. Le nombre de fleurs par inflorescence est augmenté (facteur 3 pour les inflorescences principales). Le nombre de graines récoltées est donc plus élevé.

b - Conclusion.

La croissance des plantes ayant intégré dans leur génome le gène ERG12, est, tout comme leur régénération, marquée par un caractère de précocité et de rapidité qui semble cependant légèrement s'atténuer à l'approche de la floraison et qui conduit à un important développement des inflorescences.

2. Analyses histologique et infrastructurale

Cette étude , réalisée en parallèle sur des feuilles de plantes transformées et de plantes " témoin H₂O" , a été entreprise dans le but de rechercher si les différences morphologiques observées lors de la croissance des plantes régénérées pouvaient être reliées à des modifications de nature histologique et infrastructurale . Des coupes transversales ont été obtenues à partir de fragments provenant de plantes âgées de 6 mois.

Les observations histologiques, accompagnées de mesures sur clichés photographiques , permettent de mettre en évidence, chez les plantes transformées , une diminution de la taille moyenne des cellules du mésophylle, par rapport aux plantes " témoin H₂O". Cette diminution, de l'ordre de 20 à 30% dans tous les cas examinés, implique pour un même volume, une augmentation du nombre de cellules d'une valeur équivalente. Etant donné que les organes, en particulier les feuilles, ont une taille moyenne augmentée d'un facteur 1,5 et que les entrenoeuds ont également une longueur accrue, on peut conclure à une intense prolifération cellulaire dans l'ensemble de la plante . On constate par exemple que le parenchyme palissadique apparaît bisérié alors qu'il est unisérié dans les feuilles des témoins.

L'analyse infrastructurale des cellules du

mésophylle fait également apparaître un certain nombre de modifications, qui concerne en particulier les mitochondries et les chloroplastes. Chez les " témoins H₂O" , les plastes des cellules du parenchyme palissadique sont du type amylochloroplastes ,caractérisés par la présence de nombreuses et volumineuses lentilles d'amidon intrastromatiques . Ces lentilles sont nettement moins nombreuses et de taille beaucoup plus réduite dans les cellules des plantes transformées . On note également que chez ces dernières, les mitochondries sont nombreuses, de petite taille, de forme sphérique tandis que chez les témoins, elles paraissent plus allongées et moins nombreuses . De plus , la densité en ribosomes cytosoliques apparaît plus importante chez les plantes transformées . Tous ces caractères indiquent une activité métabolique plus intense chez les plantes transformées .

3- Dosage de la chlorophylle.

Cette analyse a été entreprise dans le but de déterminer si la couleur verte des feuilles plus intense , observée au niveau des plantes transformées par rapport à celle des plantes témoins , pouvait être le reflet de modifications de la teneur en chlorophylle totale (chlorophylle a et chlorophylle b).

Plusieurs séries de dosages, réalisés sur des feuilles de niveau moyen provenant de plantes transformées et de plantes " témoin H₂O" permettent de mettre en évidence une augmentation du taux de chlorophylle totale (valeurs ramenées au poids de matériel lyophilisé) d'un facteur variant de 2,5 à 3 chez les plantes transformées . Il faut toutefois noter que, lorsque l'on s'adresse à des feuilles de la partie apicale des plantes, cette différence n'est pas retrouvée .

4 - Mesure des échanges gazeux respiratoires.

Ces mesures , réalisées à l'aide de l'oxygraphe, à partir de fragments de feuilles situées à la base de plantes âgées de six mois, ont été effectuées en parallèle chez les plantes transformées et chez les plantes témoins. Elles ont permis de mettre en évidence une modification des échanges gazeux respiratoires . En effet, chez les plantes transformées, ces échanges sont augmentés d'un facteur variant de 1,6 à 2 par rapport aux plantes " témoin H₂O " et ceci pour une même surface foliaire . Ces résultats peuvent être reliés à ceux apportés par les observations ultrastructurales concernant notamment les mitochondries et indiquent une activité métabolique plus intense chez les plantes transformées .

5. Transmission héréditaire du gène .

Les plantes transformées ont donné des graines qui majoritairement permettent l'obtention de plantes résistantes à la kanamycine .

Seulement un petit nombre (inférieur à 5%) de plantes issues de ces graines redevient sensible .

REVENDICATIONS

1. Plante caractérisée en ce qu'elle porte dans son génome un ou plusieurs gènes codant pour une ou plusieurs enzymes des étapes précoces de la voie de biosynthèse des phytostérols , lesdits gènes soit ne se retrouvant pas dans la plante native, soit étant originaires de ladite plante, auquel cas ils sont présents dans un nombre de copies supérieur à celui de la plante native ou ils sont modifiés dans leur structure ou leur fonction par rapport à ceux de la plante native.

2. Plante selon la revendication 1, caractérisée en ce que ladite étape précoce est la transformation de l'acide mévalonique en mévalonyl 5-phosphate .

3. Plante selon l'une des revendications 1 et 2, caractérisée en ce qu'elle porte un ou plusieurs gènes codant pour une ou plusieurs mévalonate-kinases.

4. Plante selon la revendication 3, caractérisée en ce que la mévalonate kinase est codée par le gène ERG12, l'un de ses dérivés ou fragments exprimant une activité mévalonate kinase.

5. Plante selon l'une quelconque des revendications 1 à 3 caractérisée en ce qu'elle porte un ou plusieurs gènes codant pour l'activité mévalonate kinase chez les crucifères , et en particulier chez Arabidopsis ou chez le colza.

6. Plante selon la revendication 1, caractérisée en ce qu'elle porte un ou plusieurs gènes codant pour une farnésyl diphosphate synthétase.

7. Plante selon la revendication 6, caractérisée en ce que la farnésyl diphosphate synthétase est codée par le gène ERG20 de la levure , ou l'un de ses dérivés ou fragments exprimant une activité farnésyl diphosphate synthétase.

8. Plante selon la revendication 1, caractérisée en ce qu'elle porte un ou plusieurs gènes codant pour une AMVP kinase.

5 9. Plante selon la revendication 8, caractérisée en ce que l'AMVP kinase est codée par le gène ERG8, ou l'un de ses dérivés ou fragments exprimant une activité AMVP kinase.

10 10. Plante selon l'une des revendications 1 à 9, caractérisée en ce qu'elle appartient à la catégorie des plantes oléoprotéagineuses .

11. Plante selon l'une des revendications 1 à 10, caractérisée en ce qu'elle appartient à la famille des crucifères et en particulier fait partie des espèces colza.

15 12. Plante selon l'une des revendications 1 à 10, caractérisée en ce qu'elle fait partie de l'espèce tournesol.

20 13. Plante selon l'une des revendications 1 à 9, caractérisée en ce qu'elle fait partie des espèces *Vigna radiata* ou aubergine.

14. Plante selon l'une des revendications 1 à 10, caractérisée en ce qu'elle appartient à la famille des Fabacées et en particulier fait partie de l'espèce soja.

25 15. Plante selon l'une des revendications 1 à 14, caractérisée en ce que le ou les gènes sont placés sous le contrôle d'un promoteur inséré à proximité du ou des gènes d'une manière à permettre l'expression de leurs produits biologiquement actifs .

30 16. Vecteur pour l'insertion d'au moins un gène codant pour une ou plusieurs enzymes des étapes précoces de la voie de biosynthèse des phytostéroïls, dans des cellules végétales, caractérisé en ce qu'il comprend :

35 - une séquence du gène à insérer,

- une ou plusieurs séquences promoteur en amont du gène à insérer ,

- une ou plusieurs séquences terminateur en aval du gène à insérer ,

5 - un ou plusieurs marqueurs permettant la sélection des cellules dans lesquelles le gène s'est inséré , et

 - éventuellement des séquences d'un plasmide Ti permettant le transfert desdits gènes et marqueurs
10 d'une bactérie vers une cellule végétale , lesdits gènes et marqueurs étant orientés et insérés , de manière à permettre leur expression.

17. Vecteur selon la revendication 16, caractérisé en ce que le gène code pour une mévalonate kinase

15 18. Vecteur selon la revendication 17, caractérisé en ce que le gène est le gène ERG12.

 19. Plasmide pFAB2 selon la revendication 18 , caractérisé en ce qu'il a été déposé sous le n° I-1176 du 14 Février 1992 auprès de la Collection Nationale
20 de Culture des Microorganismes de l'Institut Pasteur (CNCM).

 20. Procédé de traitement d'une plante par insertion dans son génome d'un gène codant pour une ou plusieurs enzymes des étapes précoces de la voie de
25 synthèse des phytostéroïls .

 21. Procédé selon la revendication 20 , caractérisé en ce que l'insertion est effectuée par transfert dans des cellules individualisées ou dans des groupes de cellules d'un vecteur selon l'une des
30 revendications 16 à 19 , éventuellement en présence d'un autre vecteur de type plasmide Ti ayant des fonctions permettant ce transfert .



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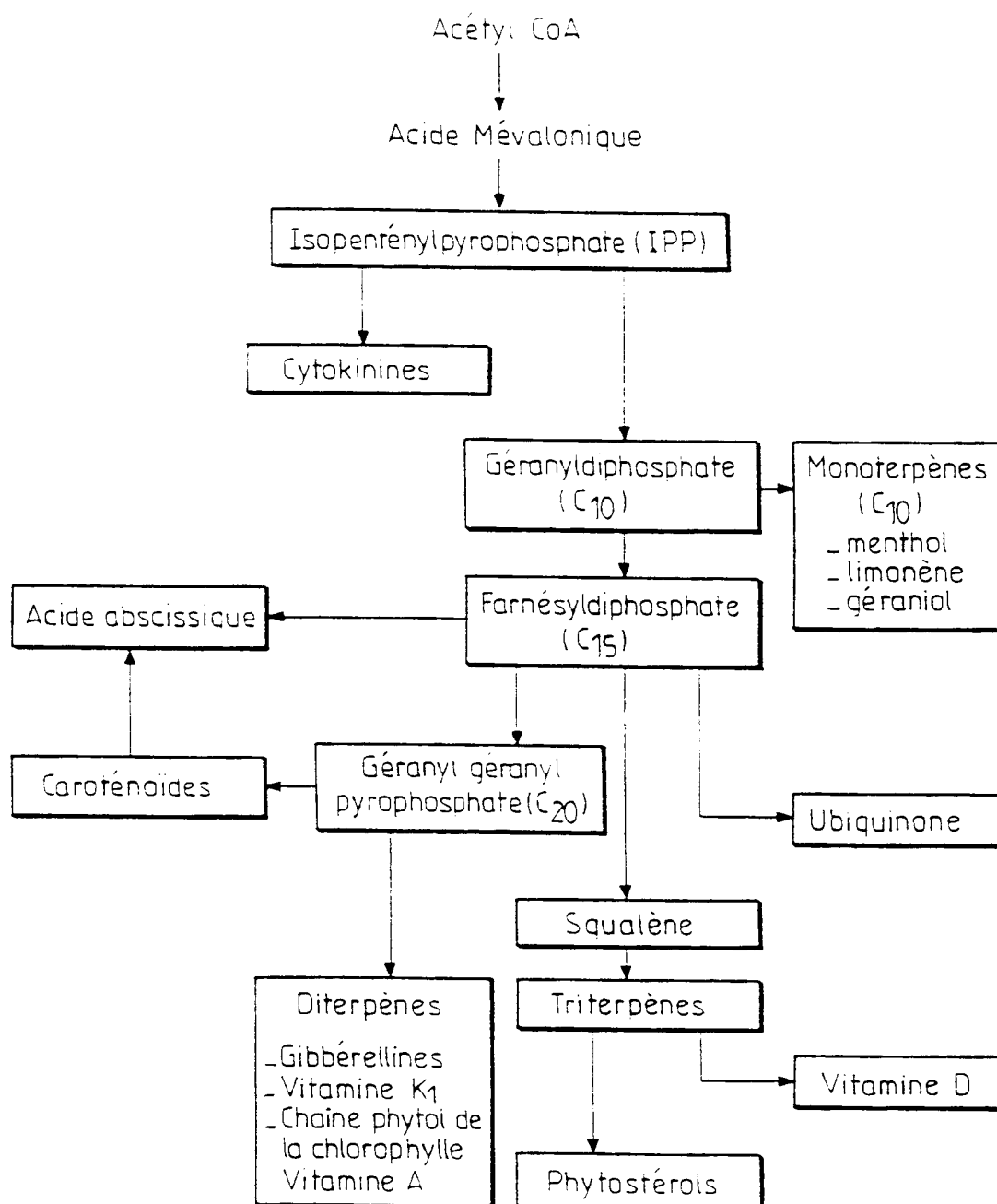


FIG. 1A



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FEUILLE DE REMPLACEMENT



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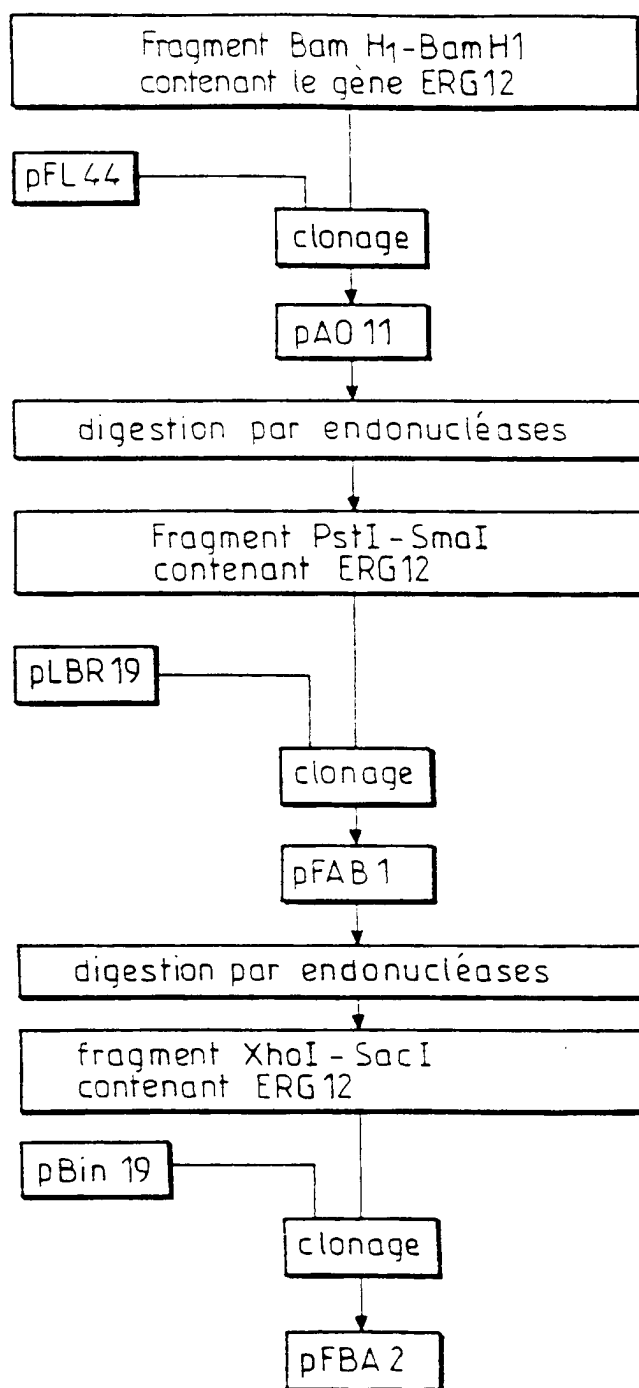
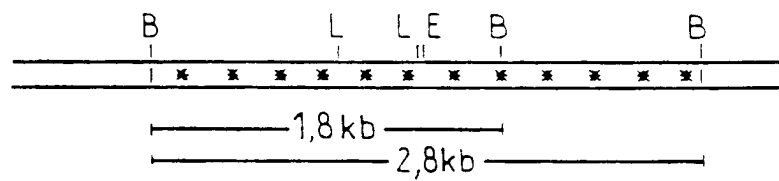




FIG. 2



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0,35 kb
 pFL44 insert contenant le gène ERG12

B, BamHI; L, BglII; E, EcoRI

FIG. 3



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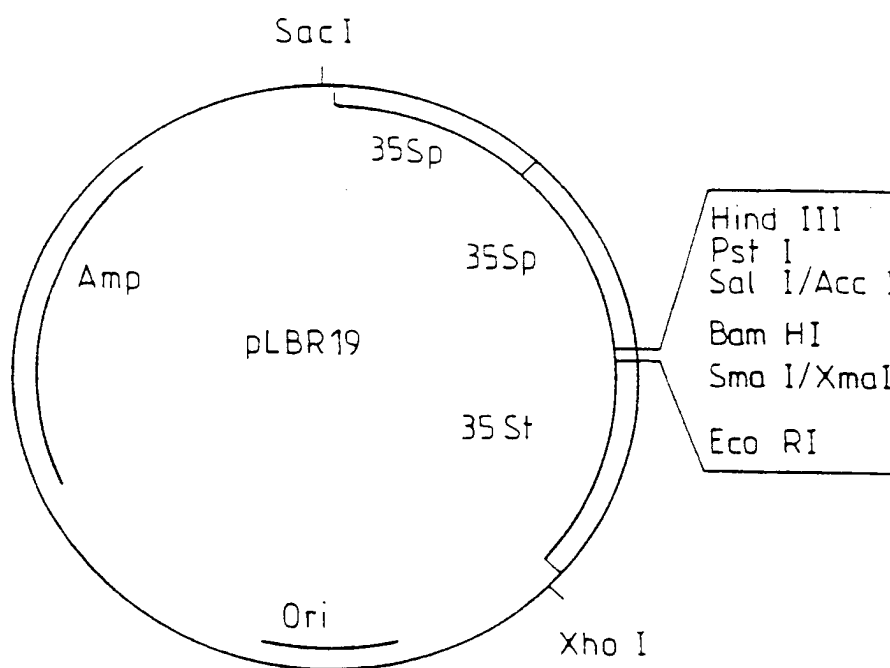


FIG. 4



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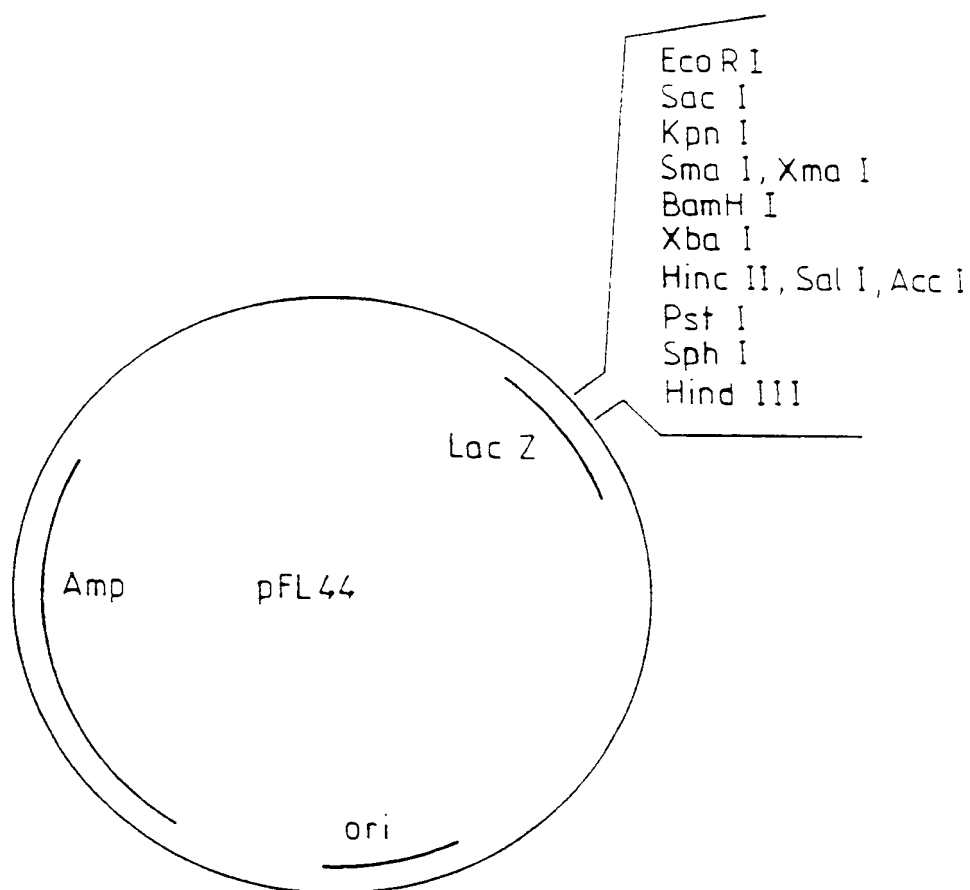


FIG. 5

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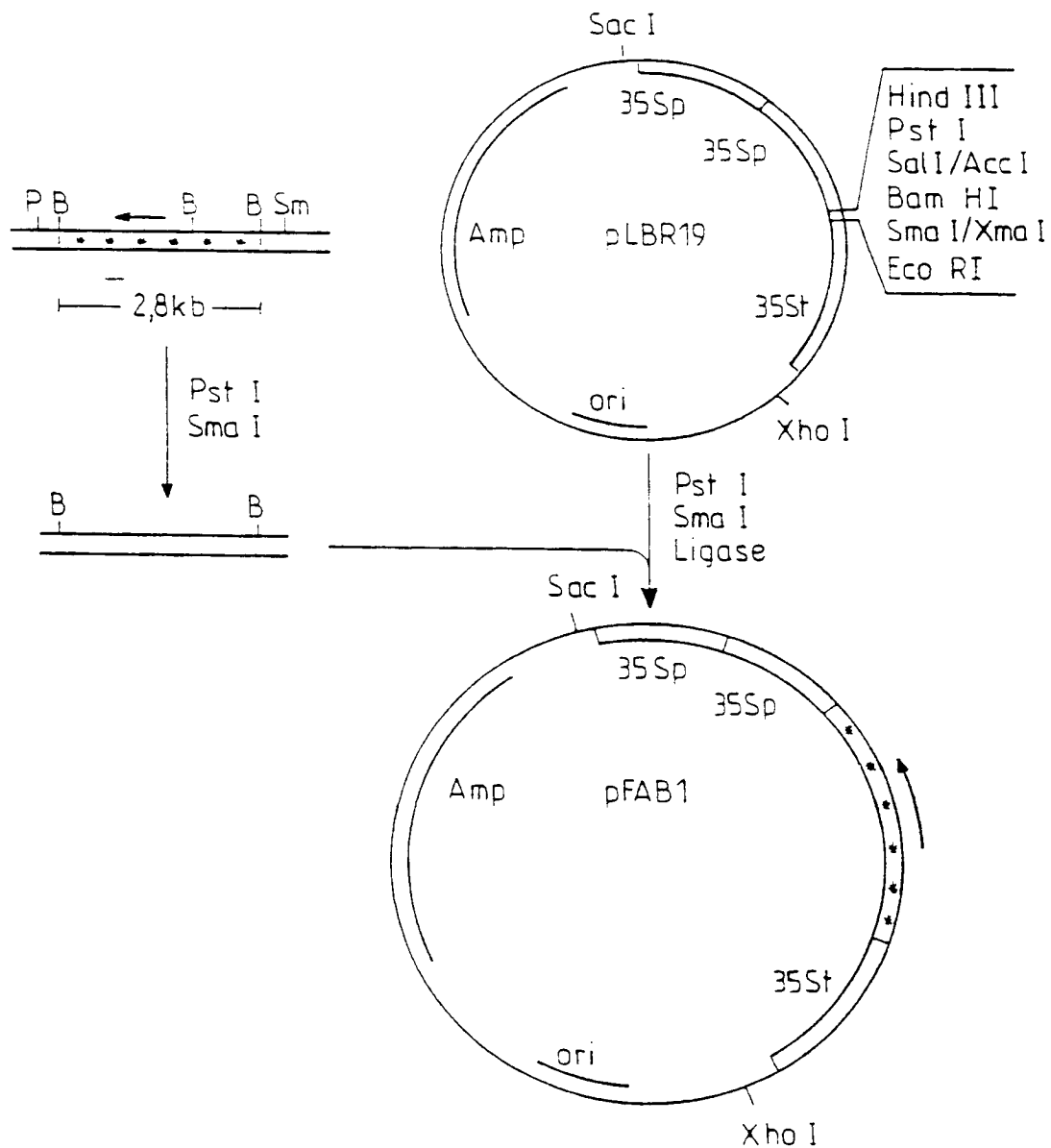


FIG. 6

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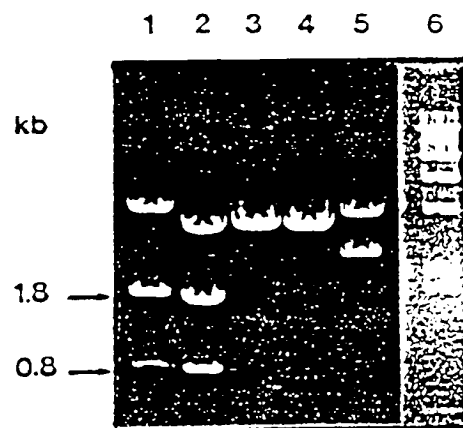


FIG.7

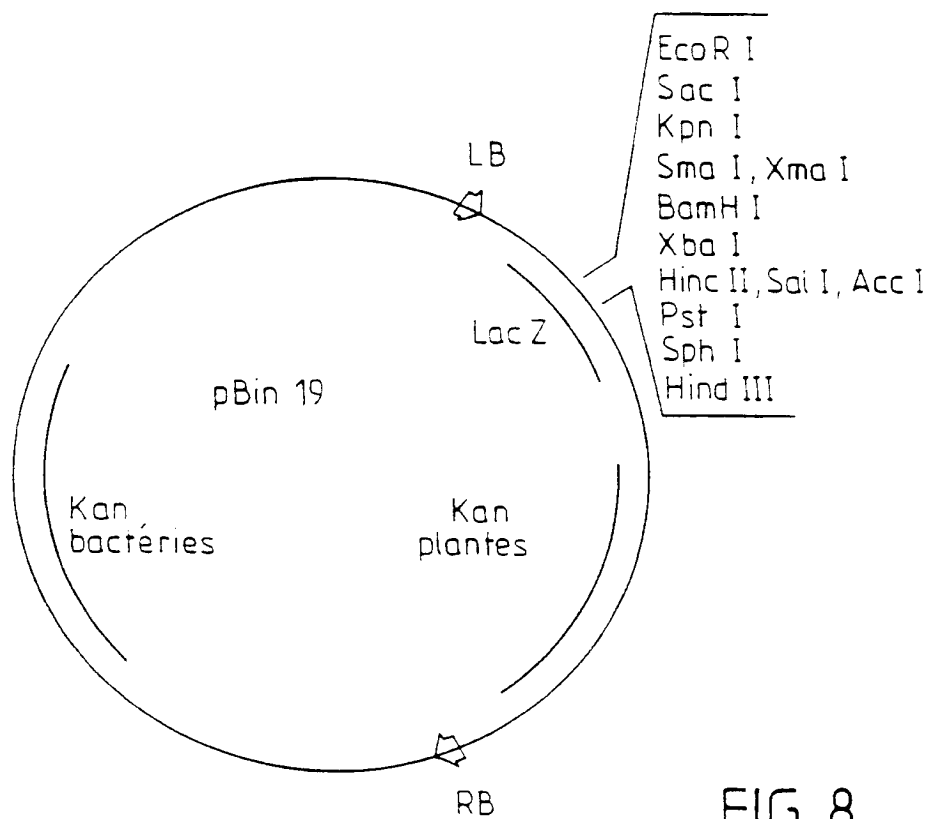


FIG.8



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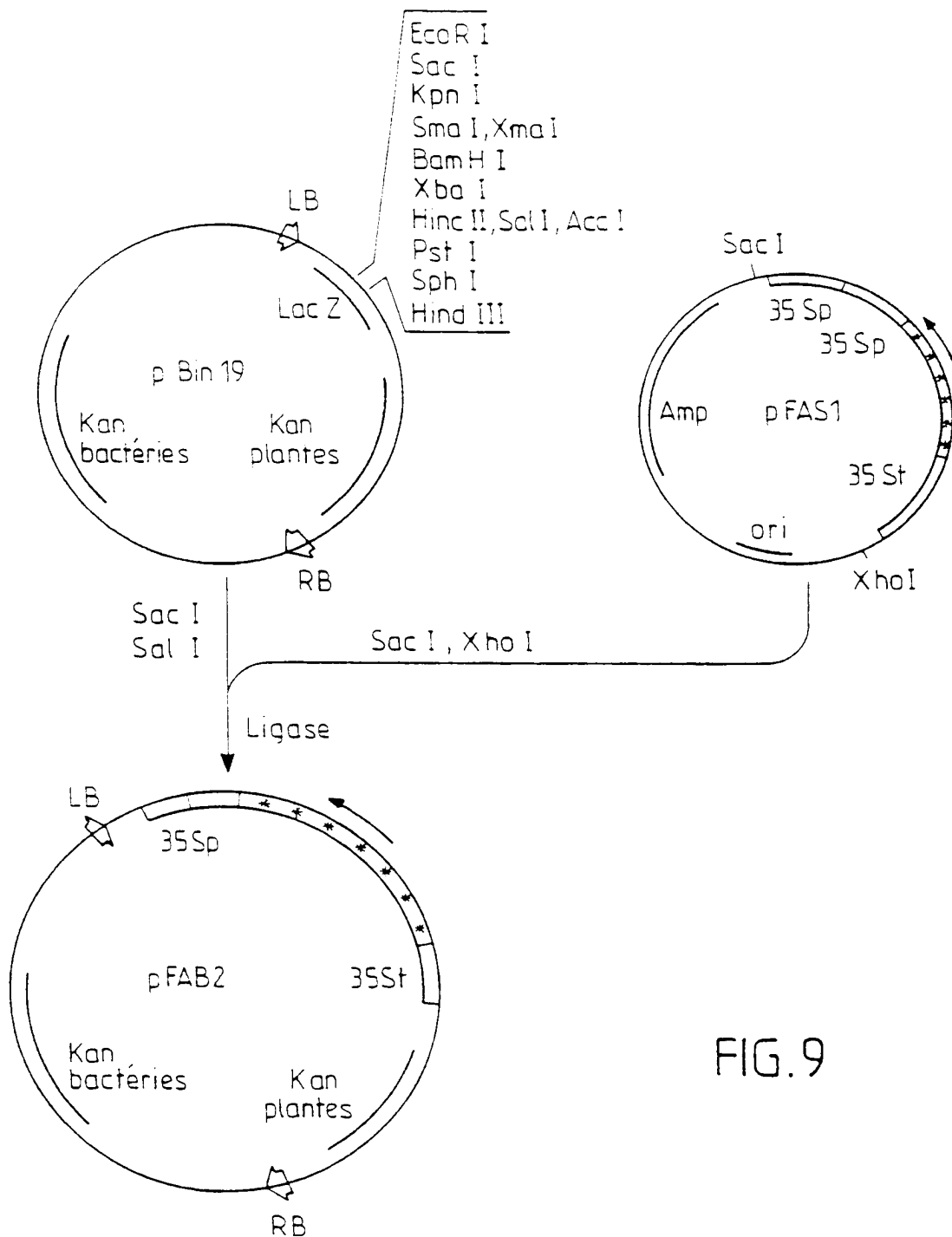


FIG.9



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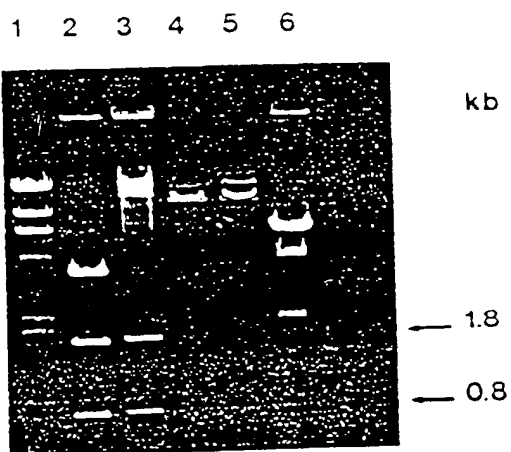


FIG.10



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FIG.11A

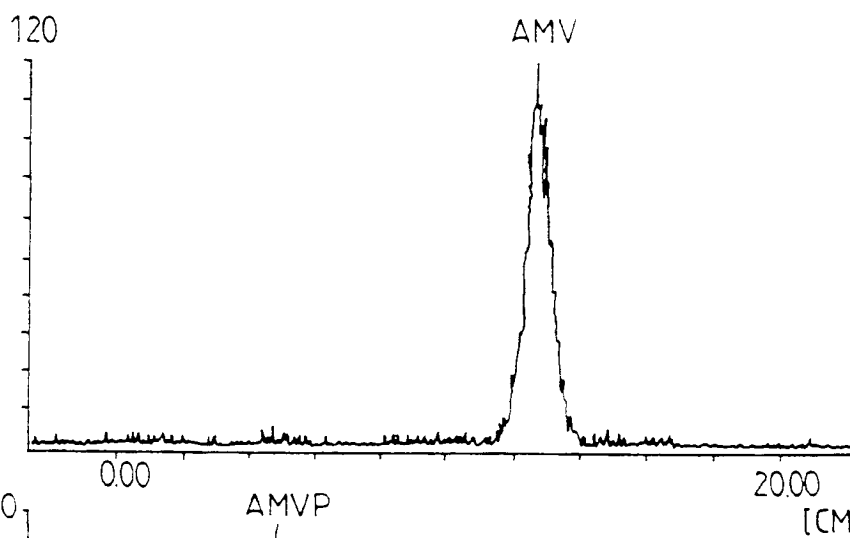


FIG.11C

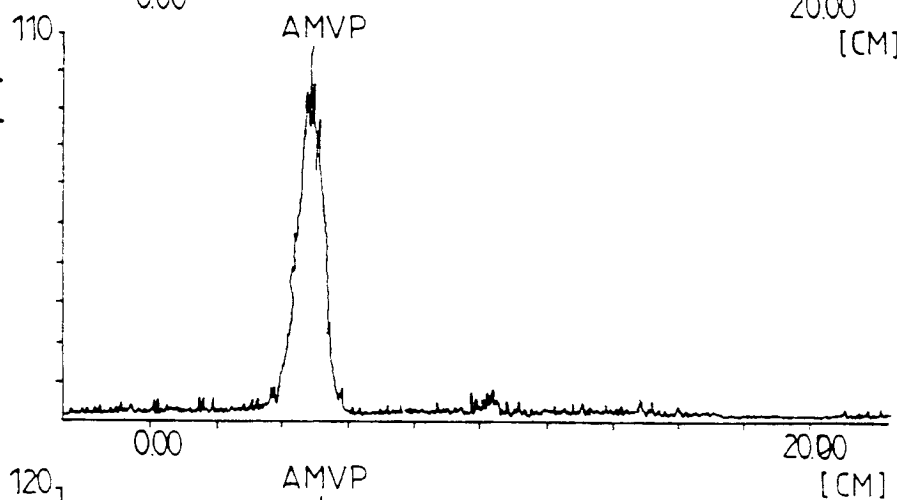


FIG.11B

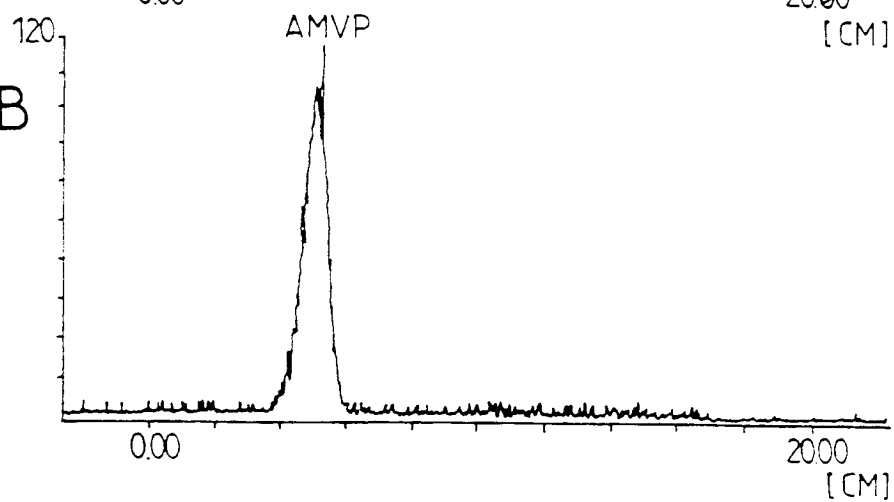


FIG.11



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FIG.12 A

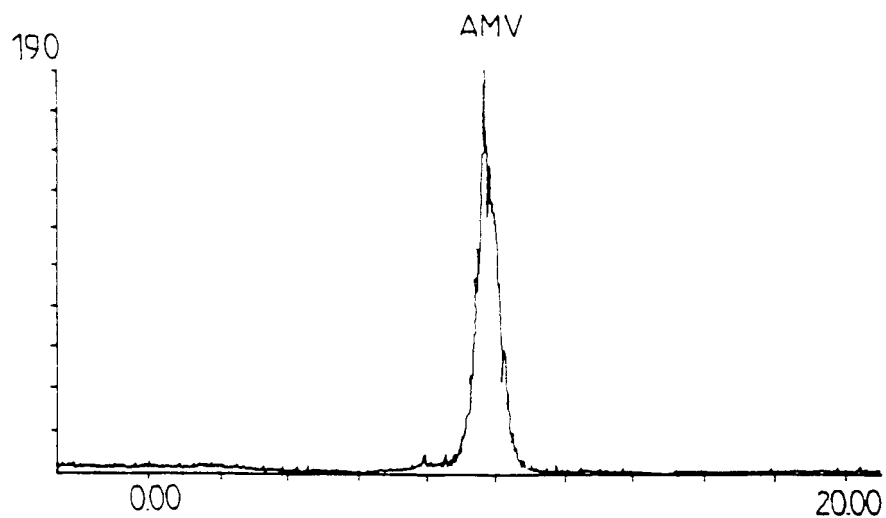
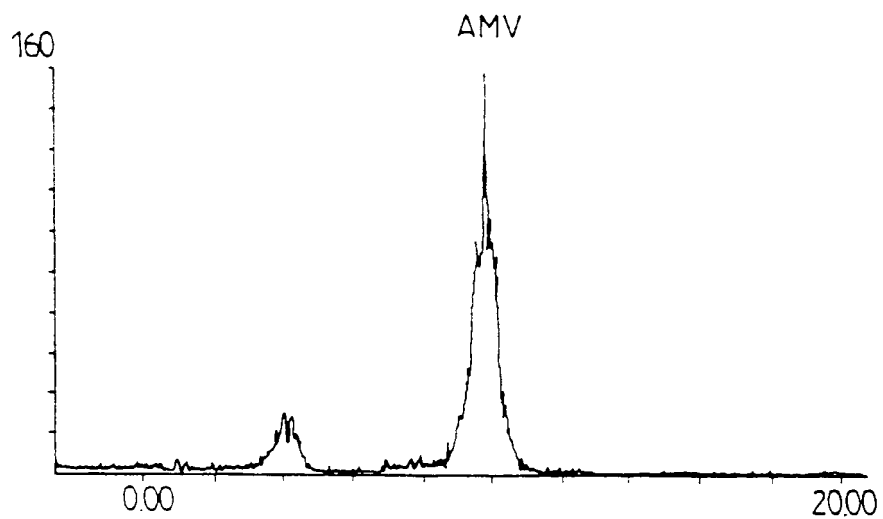


FIG.12 B





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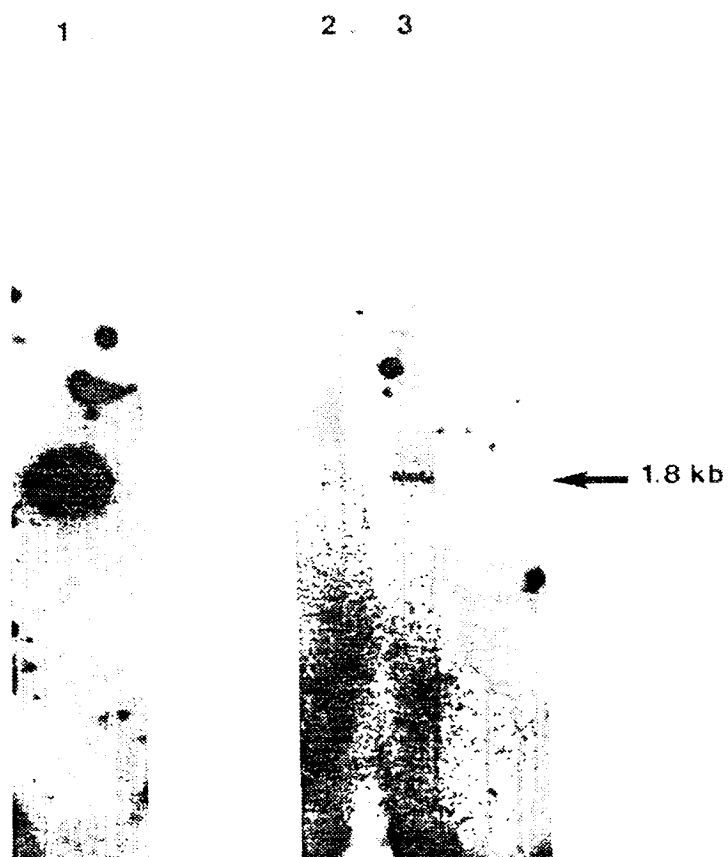
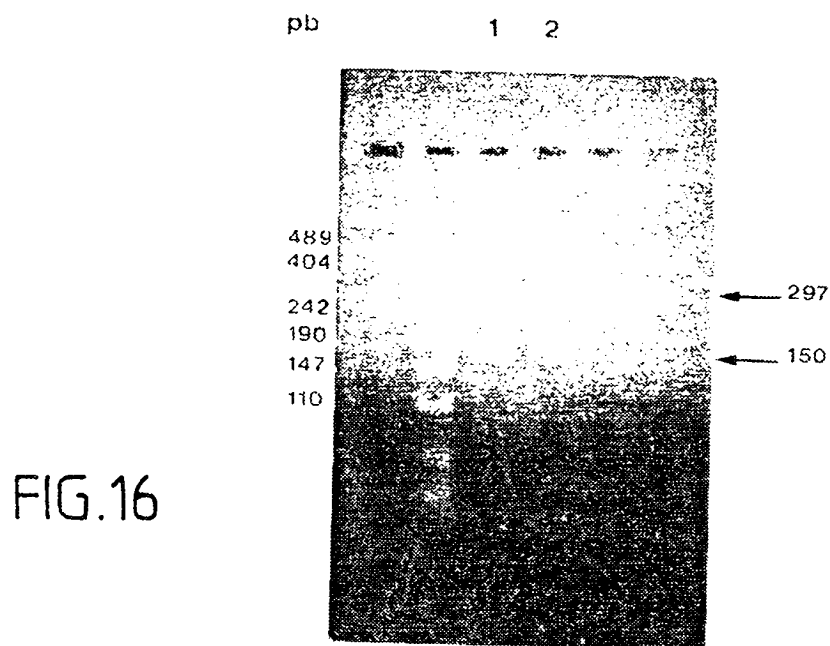
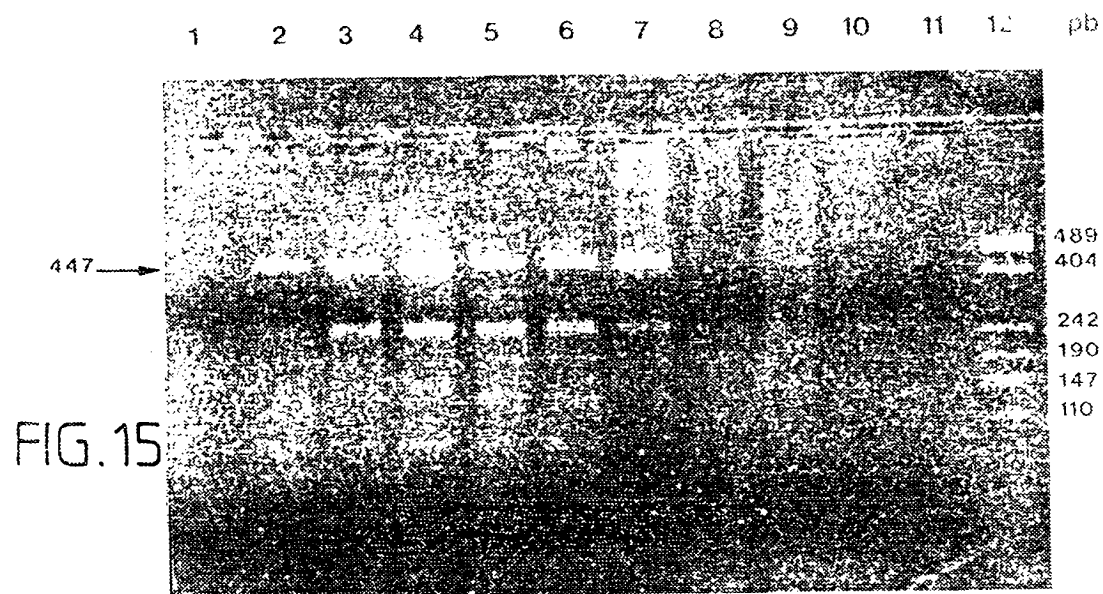


FIG.13



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FEUILLE DE REMPLACEMENT



INTERNATIONAL SEARCH REPORT

International application No.

PCT/FR 93/00134

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl.⁵ C 12 N 15/82 ; C 12 N 15/54; A 01 H 1/00; A 01 H 5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.⁵ C 12 N; A 01 H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|--|-----------------------|
| X | PLANT PHYSIOLOGY. ANNUAL MEETING OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ALBUQUERQUE, NEW MEXICO, JULY 28 - AUGUST 1, 1991. volume 96, No 1, May 1991 (ROCKVILLE, MD, USA. page 127 CHAPPELL, J., ET AL. "Is HMG-Co A reductase a rate limiting step for isoprenoid metabolism?" *abstract No 853* | 1,15,16,20,21 |
| P,X | EP, A, 0 480 730 (AMOCO) 15 April 1992 see the whole document | 1,15,16,20,21 |
| A | EP, A, 0 409 628 (CALGENE) 23 January 1991 see the whole document | 1,15,16,20,21 |
| -/-- | | |

☐ Further documents are listed in the continuation of Box C☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

8 July 1993 (08.07.93)

Date of mailing of the international search report

21 July 1993 (21.07.93)

Name and mailing address of the ISA

European Patent office

Facsimile No.

Authorized officer

Telephone No.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FR 93/00134

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passage | Relevant to claim(s) |
|-----------|--|----------------------|
| A | WO, A, 9 113 078 (AMOCO) 5 September 1991 cited in the application see page 104 - page 106 ----- | 1, 15, 16, 20, 21 |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

FR 9300134
SA 70502

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

| | | |
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| I. CLASSEMENT DE L'INVENTION (si plusieurs symboles de classification sont applicables, les indiquer tous) ⁷ | | |
| Selon la classification internationale des brevets (CIB) ou à la fois selon la classification nationale et la CIB | | |
| CIB 5 C12N15/82; | C12N15/54; | A01H1/00; A01H5/00 |
| II. DOMAINES SUR LESQUELS LA RECHERCHE A PORTE | | |
| Documentation minimale consultée ⁸ | | |
| Système de classification | Symboles de classification | |
| CIB 5 | C12N ; A01H | |
| Documentation consultée autre que la documentation minimale dans la mesure où de tels documents font partie des domaines sur lesquels la recherche a porté ⁹ | | |
| III. DOCUMENTS CONSIDERES COMME PERTINENTS ¹⁰ | | |
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| ^o Catégories spéciales de documents cités: ¹¹ "A" document définissant l'état général de la technique, non considéré comme particulièrement pertinent "E" document antérieur, mais publié à la date de dépôt international ou après cette date "L" document pouvant jeter un doute sur une revendication de priorité ou cité pour déterminer la date de publication d'une autre citation ou pour une raison spéciale (telle qu'indiquée) "O" document se référant à une divulgation orale, à un usage, à une exposition ou tous autres moyens "P" document publié avant la date de dépôt international, mais postérieurement à la date de priorité revendiquée "T" document ultérieur publié postérieurement à la date de dépôt international ou à la date de priorité et n'appartenant pas à l'état de la technique pertinent, mais cité pour comprendre le principe ou la théorie constituant la base de l'invention "X" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme nouvelle ou comme impliquant une activité inventive "Y" document particulièrement pertinent; l'invention revendiquée ne peut être considérée comme impliquant une activité inventive lorsque le document est associé à un ou plusieurs autres documents de même nature, cette combinaison étant évidente pour une personne du métier. "&" document qui fait partie de la même famille de brevets | | |
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